GENERATION OF CLONAL MICROPLANTS AND HAIRY ROOT CULTURES OF THE AROMATIC MEDICINAL PLANT *Salvia runcinata* L.f.

by

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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own original work, that I am the sole author thereof (unless to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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Summary

Bacterial and fungal pathogens have developed numerous defence mechanisms against antimicrobial chemical agents, and resistance to old and new produced drugs are on the rise. Discovery of natural products derived from plants with diverse chemical structures and novel mechanisms of action to treat these notorious pathogens is a priority. Biotechnology (discussed in **Chapter 1**) has much to offer as a pharmacological tool and in the general study of medicinal plants. The Genus *Salvia* (Lamiaceae) has gathered much interest as these plants manufacture a diverse range of secondary metabolites including flavonoids, tannins and terpenoids. Of particular interest are the terpenoids which are largely implicated in the efficacy of *Salvia* plants as traditional medicines contributing to their pharmacological actions (discussed in **Chapter 2**). Due to the importance of these plants as herbal remedies, in this study, biotechnological techniques such as tissue culture and *Agrobacterium*-mediated transformation were applied on *Salvia runcinata* L.f., a South African medicinal plant, in an attempt to enhance the metabolomic profile and its bioactivity. Like so many other sages, *S. runcinata* has been used in folk medicine to treat a variety of ailments. Application of biotechnology was viewed as an important value adding platform for this species, assisting with its commercialisation for the cosmeceutical and pharmaceutical industries. Therefore the study had three foci: (1) to determine the seed germination behaviour and optimal conditions for micropropagation; (2) to develop a protocol that would be efficient whilst being simple for genetic transformation; and lastly, (3) to conduct phytochemical studies on *in vitro* generated *S. runcinata* transgenic hairy root and *in vitro* organ cultures by comparing these to glasshouse plants as potential therapeutic sources of natural compounds used in the treatment of infections in plants and humans.

Data generated is thus summarised in three research chapters and **Chapter 3** describes the formulated procedures assisting with *in vitro* seed germination and micropropagation of *S. runcinata*. The efficacy of smoke and scarification treatments for germination improvement was initially tested coupled to the evaluation of different hormonal combinations and different explant types which would aid with inducing

adventitious shoot formation *in vitro*. The most effective germination treatment proved to be a 3 min exposure of seeds to 25% (w/v) H_2SO_4 combined with a concentration of 10^{-5} M smoke solution, resulting to more than 80% germination. Shoot proliferation was significantly higher using nodal explants with the addition of 4.43 µM BA. The protocol established in this part of the study is viable for large scale commercial production of *S. runcinata* as it would yield 1296 to 46656 viable plants in 4 to 6 months from one nodal explant. Micropropagation was applied also as a pre-emptive measure to ease pressure on the wild plants as the demand for *S. runcinata* is anticipated to increase due to its growing economic value as it is one of two South African sages with epi-α-bisabolol that is sought after by the pharmaceutical and cosmeceutical industries. This makes the protocol developed in this part of the study suitable for *ex situ* conservation of *S. runcinata* plantlets.

Evaluations on the transgene transfer capacities of two different agropine strains (A4T and LBA 9402) of *Agrobacterium rhizogenes* to induce hairy root cultures of *S. runcinata* explants on nodal and leaf explants were conducted (reported in **Chapter 4**). Hairy roots formed 3 to 4 weeks after inoculation of the explants and these agropine strains showed different abilities for genetic transformation with the LBA 9402 strain producing significantly more roots on each explant compared to the A4T strain (P=0.0075). However, none of the LBA 9402 derived clones and only 2 clones generated through A4T transformation survived subculturing. The polymerase chain reaction (PCR) and reverse transcriptase-polymerase chain reaction (RT-PCR) confirmed the presence and transcription (respectively) of *rol* A, *rol* B, *rol* C and *ags* genes which are mobilised from the transfer-DNA (T-DNA) fragment of the root-inducing (Ri) plasmid of *A. rhizogenes* to the plant genome during transformation. The two A4T clones, termed here A4T3 and A4T5, were stably transformed, Southern blot analysis using *rol* A as a probe further validated the integration of one copy of the *rol* A gene.

Transformed hairy roots, untransformed roots from tissue cultured plants, tissue culture-derived plants and glasshouse-grown plants were profiled for secondary metabolites by thin layer chromatography (TLC) and gas chromatography-mass

spectrometry (GC-MS) in **Chapter 5**. In this part of the study, it is clear that the use of tissue culture as a propagation system did not negatively affect the volatile compound profile of *S. runcinata* and plants had a similar essential oil content to that reported by Kamatou *et al.* (2008), leading to a conclusion that *in vitro* plants maintained their biochemical integrity even under an alternative micro-controlled environment. Similarly to others, Ri-transformation was explored as an avenue to alter secondary metabolism creating inter-clonal variation. Transformed clones were distinguishable, displaying more of some primary metabolites including sucrose, galactose, sorbose and fructose than the leaf extracts. With the current GC-MS methods used, this clear distinction was not obvious at the secondary metabolite level.

In general, solvent extracts (acetone and methanol:dichloromethane (MetOH: DCM) (1:1 v/v) exhibited good to moderate antibacterial activity with the minimum inhibitory concentration (MIC) values ranging from 0.39 to 0.78 mg ml^{-1}. However, *in vitro* plant cultures were the most potent against two Gram-negative bacterial strains: *Escherichia coli* (ATCC 11775) and *Klebsiella pneumoniae* (ATCC 13883), and two Gram-positive bacterial strains: *Bacillus subtilis* (ATCC 6051) and *Staphylococcus aureus* (ATCC 12600). The hairy root extracts did not show any activity against fungi, *Fusarium subglutinans* (MRC 0115) and *Fusarium proliferatum* (MRC 6908).

Micropropagation therefore proves to be an interesting avenue for commercial production of *S. runcinata*, supplying plants with an improved pharmacological activity. Hence the biotechnological approach applied here is a viable strategy for the production of medicinal bioactives from *S. runcinata*.

Opsomming

Bakterieë en fungi patogene het baie verskeie meganismes ontwikkel teen antimikrobiese chemiese agente, en weerstand teen ou en nuwe chemise stowwe is besig om te vergroot. Daarom is dit belangrik om natuurlike plantaardige produkte met diverse chemiese strukture en unieke werkings meganismes te ontdek waarmee hierdie berugte patogene beveg kan word. Biotegnologie (wat in **Hoofstuk 1** bespreek word) kan gebruik word as ’n farmakologiese hulpmiddel in die algemene studie van plante. Die Klas (Genus) *Salvia* (Lamiaceae) het al baie aandag getrek aangesien hierdie plante ’n wye reeks sekondêre metaboliete vervaardig wat flavonoïede, tanniene en terpenoïede insluit. Veral van belang is die terpenoïde wat betrokke is by die doeltreffendheid van die *Salvia* plante as tradisionele medisyne, aangesien dit bydra tot hulle farmalogiese aksie (wat in **Hoofstuk 2** bespreek word). Aangesien hierdie plante sulke belangrike kruie is, word daar in hierdie studie, biotegnologiese tegnieke soos die kweek van weefsel en *Agrobacterium-bemiddelde* transformasie op *Salvia runcinata L.f.* toegepas om die metabologiese profiel en die bioaktiwiteit daarvan te verbeter. Soos baie van die salies is *S. runcinata* tradisioneel dikwels gebruik om allerhande siektetoestande te behandel. Die toepassing van biotegnologie word beskou as ’n belangrike manier om waarde by te voeg sodat hierdie plant kommersieei deur die kosmetiese en farmakeutiese bedrywe gebruik kan word. Daarom is daar op drie dinge gefokus: (1) die ontkiemings gedrag van saad en die optimale toestande vir mikrovoortplanting (2) die ontwikkeling van protokol wat eenvoudig maar doeltreffend is vir genetiese transformasie, en die (3) fito-chemise studies op *in vitro* genereerde *S. runcinata* transgeniese harige wortels en *in vitro* orgaan kwekings deur om hulle te vergelyk met kweekhuis plante as potentiële terapeutiese bronne van natuurlike samestellings vir die behandeling van infeksies in beide plante en mense.

Die data wat gegenereer is, is opgesom in drie hoofstukke, en in **Hoofstuk 3** word die prosedures wat gebruik word in die *in vitro* saad ontkieming en die mikro voortplanting van *S. runcinata,* bespreek. Die doeltreffendheid van rook en skarifikasie behandeling vir die verbetering van ontkieming is eers getoets en

gekoppel aan die evaluering van verskillende hormoonkombinasies en verskillende eksplant tipes wat lei tot die formasie van uitloopsels *in vitro.* Daar is gevind dat die effektiefste behandeling vir ontkieming, ’n 3-minuut blootstelling van saad aan 25% (w/v) H_2SO_4 gekombineer met ’n konsentrasie 10^{-5} M rook oplossing is. Dit het gelei tot meer as 80% ontkieming. Daar was baie meer uitloopsels toe nodale eksplante gebruik is met die byvoeging van 4.43 µM BA. Die proktokol wat hier gevestig is, kan op groot skaal gebruik word vir die kommersiële produksie van *S. runcinata*, want 1296 tot 46656 lewensvatbare plante kan binne 4 ot 6 maande van een nodale eksplant gemaak word. Mikro voortplanting is toegepas as ’n voorkomende maatreel om die druk op die natuur te verminder omdat daar verwag word dat die vraag na *S. runcinata* sal toeneem na gelang die groeiende ekonomiese waarde daarvan toeneem. Dit is een van twee Suid-Afrikaanse salies met epi-α-bisabolol wat deur die farmakeutiese en die kosmetiese bedrywe gebruik word. Dit beteken dat die protokol wat hier ontwikkel is, geskik is vir die *ex situ* bewaring van *S. runcinata* plante.

Die transgeen oordrag van twee verskillende agropien tipes (A4T and LBA 9402) van *Agrobacterium rhizogenes* is geevalueer (en in **Hoofstuk 4** beskryf). Harige wortels het 3 tot 4 weke na die inenting van die eksplante gevorm en hierdie agropien tipes het verskillende vermoëns vir genetiese transformasie getoon, met die LBA 9402 tipe wat baie meer wortels op elke eksplant voorgebring het in vergelyking met die A4T tipe (P=0.03116). Geen van die LBA 9402-afgeleide klone en slegs 2 klone wat deur A4T transformasie genereer is, het oorleef. The polimerase ketting reaksie (PCR) en die teenoorgestelde trenskriptasie-polimerase (RT-PCR) ketting reaksie het die teenwoordigheid en transkipsie (onderskeidelik) van *rol* A, *rol* B en *rol* C en *ags* gene, wat oorgedra word deur die oordrag DNA (T-DNA) fragment van die wortel induserende (Ri) plasmied van *A. rhizogenes* na die plant genoom tydens transformasie, bevorder. A4T klone, hier A4T3 and A4T5 genoem, is stabiel transformeer. Southern blot ontleding het met die gebruik van *rol* A, die integrasie van een kopie van die *rol* A geen, bevestig.

In **Hoofstuk 5** is transformeerde harige wortels, ongetransformeerde wortels van weefsel gekweekte plante, weefsel gekweekte plante, en kweekhuis plante deur

dun-laag chromatografie (TLC) en gas-chromatografie-massa spektrometrie (GC-MS) geprofiel vir sekondêre metaboliete. In hierdie deel van die studie is dit duidelik dat die gebruik van weefsel kwekery as ’n voortplantsisteem nie ’n negatiewe effek gehad het op die vlugtige samestelling profiel van *S. runcinata nie* en dat plante ’n sootgelyke essentiële olie inhoud het as wat deur Kamatou *et al.* (2008) bevind is. Dit lei tot die gevolgtrekking dat *in vitro* plante hulle biochemiese integriteit behou selfs onder alternatiewe mikro-beheerde omgewings. Ri-transformasie is ondersoek as ’n manier om sekondêre metabolisme te verander om interkloon variasie te skep. Getransformeerde klone kon uitgeken word, aangesien dit meer primêre metaboliete soos sukrose, galaktose en fruktose insluit as die blaar ekstrakte. Hierdie verskil was nie met die huidige GC-MS metodes so duidelik sigbaar op die sekondêre metabolitiese vlak nie.

Oor die algemeen toon ekstraksie met asetoon en methanol dichlorometaan (MetOH: DCM) (1:1 v/v) goeie tot gemiddelde antibakteriese aktiwiteit met die minimum remmende konsentrasie (MIC) waardes van 0.39 tot 0.78 mg ml^{-1}. Die *in vitro* plant kulture het egter sterker weerstand gebied teen twee Gram-negatiewe bakteriese tipes: *Escherichia coli* (ATCC 11775) en *Klebsiella pneumoniae* (ATCC 13883), en teen twee Gram-positiewe bakteriese tipes: *Bacillus subtilis* (ATCC 6051) en *Staphylococcus aureus* (ATCC 12600). Die harige wortel ekstrakte het geen aktiwiteit teen die swamme, *Fusarium subglutinans* (MRC 0115) en *Fusarium proliferatum* (MRC 6908) getoon nie.

Mikro-voortplanting is dus ’n interessante manier om *S. runcinata* kommersieel te produseer aangeien die plante verbeterde farmalogiese aktiwiteit toon. Die biotegnologiese benadering wat hier toegepas word, is ’n praktiese strategie vir die produksie van geneesmiddels van *S. runcinata*.

Papers from this thesis

Submitted manuscripts:

Figlan S, Ndimande SG, Makunga NP. *Agrobacterium rhizogenes*-mediated transformation of the medicinal *Salvia runcinata* L. f. Plant Cell Tissue and Organ Culture. PCTO-S-12-005973-3-R1 (Revision) (Submitted 5th Oct 2012) IF=3.1

Figlan S, Makunga NP. Positive activity of aromatic *Salvia runcinata* (L.f.) grown *in vitro* and in the glasshouse against two highly prolific *Fusarium* species. Plant Biotechnology Reports. PBR-5-12-00381 (Submitted 1st Oct 2012) IF=1.2

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Abbreviations

$A_{260/280}$	Absorbance at 260 or 280 nm
ABA	Abscisic acid
ags	Agropine synthase gene
AIDS	Acquired immune deficiency syndrome
AMDIS	Automated mass spectral deconvolution and identification system
ANOVA	Analysis of variance
BA	6-benzyladenine (PGR)
bp	Base pairs (nucleic acid)
°C	Degrees celcius
CAF	Central analytical facility
CBC	Community-based conservation
cDNA	Copy deoxyribonucleic acid
CFU	Colony forming unit
chv	Chromosomal virulence gene
cm	Centimeter
CSIR	Council for Scientific and Industrial Research
CTAB	Cetyl trimethyl ammonium bromide
DCM	Dichloromethane
dH_2O	Distilled water
ddH_2O	Deionised water
DNA	Deoxyribonucleic acid
dATP	Deoxyadenosine triphosphate
dNTP's	Deoxyribonucleotide triphosphates
DST	Department of science and technology
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
=	Equal to
Ex vitro	Cultivated in the natural conditions

FAO	Food and Agriculture Organisation
$FeSO_4$	Ferrous sulfate
F_{254}	Fluorescent indicator with a 254 nm excitation wavelength
g	Gram
GA	Gibberelic acid
GC-MS	Gas chromatography mass spectrometry
GH	Glasshouse
g L^{-1}	Grams per litre
g ml^{-1}	Grams per milliliter
≥	Greater than or equal to
GM	Genetic modification
h	Hour
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
HL-60	Human leukemia-60 cells
H_2SO_4	Sulphuric acid
IAA	Indole-3-acetic acid (PGR)
INR	Institute for Natural Resources
INT	*p*-iodonitrotetrazolium violet
In vitro	“in glass”
IOCD	International Organisation for Chemical Sciences
IUCN	International Union for Conservation of Nature
IUFRO	International Union of Forest Research Organisation
Kar_1	Karrikin 1
kb	Kilo base pair
KCl	Potassium chloride
kg	Kilogram
KH_2PO_4	Monopotassium phosphate
KNO_3	Potassium nitrate
kPa	Kilo pascal
L	Litre
LSD	Least significant difference
µ	Micro

M	Molar
MAPs	Medicinal and aromatic plants
µCi/mmol	Microcurie per millimolar
MCF-7	Michigan cancer foundation - 7
MeOH	Methanol
µg	Microgram (10^{-6} g)
µg ml^{-1}	Microgram per milliliter
mg	Milligram (10^{-3} g)
mg l^{-1}	Milligram per liter
mg ml^{-1}	Milligram per milliliter
$MgSO_4$	Magnesium sulfate
MH	Müller-Hinton
MIC	Minimum inhibitory concentration
min	Minute
mJ cm^{-1}	Millijoule per centimeter
µl	Microliter (10^{-6} L)
ml	Milliliter (10^{-3} L)
µM	Micromolar (10^{-6} M)
µmol m^{-1} s^{-1}	Micromole per meter per second
mm	Millimeter
$MnSO_4$	Manganese sulphate
MRC	Medical Research Council
MS	Murashige and Skoog (1962) medium
½ MS	Half-strength MS medium
m/z	Mass-to-change ratio
N	Sample size
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NH_4OH	Ammonium hydroxide
NIST	National Institute of Standards and Technology
nm	Nanometre
no.	Number
OD_{600}	Optical density at 600 nm

ORF	Open reading frame
PAR	Photosynthetic active radiation
%	Percent
PCR	Polymerase chain reaction
PGR	Plant growth regulator
±	Plus / minus
pH	Measure of acidity and alkalinity
PPFD	Photosynthetic photon flux density
ppm	Parts per million
Pty Ltd	Proprietary limited company
PVP	Polyvinylpyrrolidone (water-soluble)
®	Registered
rcf	Relative centrifugal force
Ri	Root inducing
Rol	Root locus
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase-polymerase chain reaction
SANBI	South African National Biodiversity Institute
sec	Second
SDS	Sodium dodecyl sulphate
SMME	Small-, micro- and medium-sized enterprises
SPME	Solid-phase microextraction
spp.	Species in plural form
SSC	Saline sodium citrate
Taq	*Thermus aquaticus*
TBE	Tris-borate EDTA
TC	Tissue culture
T-DNA	Transfer DNA
TE buffer	Tris-EDTA buffer
Ti	Tumor inducing
T_L	Left T-DNA region
TLC	Thin layer chromatography

TM	Trade mark
TMS	Trimethlychlorosilane:hexamethyldisilazane:pyridine
TPC	Total phenolic content
t_R	Retention time
T_R	Right T-DNA region
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
U	Unit
UNICEF	United Nations Children's Emergency Fund
US	United States
USA	United States of America
UV	Ultra violet
V	Volts
vir	Virulence
v/v	Volume to volume ratio
WHO	World health organisation
w/v	Mass per volume ratio
WWF	World Wildlife Fund
YEP	Yeast extract peptone
$ZnSO_4$	Zinc sulphate

Chapter 1

Introduction

Biotechnology is an important tool for the manipulation of genes, thus "tricking" plants into producing novel products, enhancing their quality to better suit the needs of Man (Nigro *et al.,* 2004). This technology has emerged as an independent and exciting discipline that is drawing worldwide attention from governments and the corporate world because of its limitless applications. In 2000, the South African government began to focus on, and substantially increased, its research support for biotechnology. This led to the adoption of the 2001 National Biotechnology Strategy (Msomi, 2008), a policy framework to create incentives for the biotechnology sector, involving several government departments (Cloete *et al.,* 2006; Moyo *et al.,* 2011).

Tracing the technology's history; a Hungarian agricultural engineer, Karl Ereky was the person to come up with the term 'Biotechnology' in 1917 defining 'all ranks of works where there was a large scale production of products from raw material with the aid of living things', but it was later redefined in 1961 because of advances in technology and discovery of new applications (Rastogi, 2007). According to Rastogi (2007) biotechnology is defined as the industrial use of microorganisms and living plant and animal cells to produce substances or effects beneficial to mankind. This technology has evolved from focusing on processing food for humans and livestock to a technology that has a very different meaning in the eyes of the public. Scientists have shifted to genetic modification (GM) or genetic engineering in the 1980's. These technologies created unprecedented opportunities for the manipulation of biological systems (Hopkins, 2007). Through these technologies, we can now directly modify the expression of genes related to natural product biosynthesis (Saito *et al.,* 1992).

For the purpose of this thesis, the use of these technologies in the context of plant tissue culture and *Agrobacterium*–mediated transformation is discussed in depth, looking at solving problems that are inherent in the production of medicinal plants such as genetic and phenotypic variability, variability and instability of extracts, toxic components and contaminants (Canter *et al.,* 2005). Consequently the use of these technologies better enhances the quality of these medicinal plants and through *Agrobacterium*-mediated transformation, enormous possibilities of upregulating high-value secondary metabolites in plant cells are provided.

There has been significant progress in the use of these two technologies to alter pathways for the biosynthesis of target metabolites (Canter *et al.,* 2005). However, a long-term accumulation of a basic understanding of chemistry, biochemistry and molecular biology of the plant secondary metabolites biosynthesis is an essential prerequisite (Saito *et al.,* 1992). In the past 20 years, researchers have put some effort in attempting to understand the characterisation of plant secondary metabolite pathways at the level of biosynthetic intermediation and enzymes (Verpoorte and Memelink, 2002). So far, the flavonoid biosynthetic pathway is the one that has been extensively studied using genetic, biochemical and molecular approaches (Winkel-Shirley, 2001).

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Chapter 2

Literature review

2.1. A review on the importance of medicinal and aromatic plants (MAPs) in southern Africa

2.1.1. Use of MAPs in southern Africa

According to Bogers *et al*. (2006), medicinal and aromatic plants (MAPs) are plants which are primarily used for medical or aromatic purposes in pharmacy and perfumery. These plants are key sources for plant secondary metabolites, which are imperative for, or play a central role in human health-care (Kumar *et al.,* 2008). Various medicinal plants have been used for years in daily life to treat diseases all over the world. Interest in medicinal plants reflects the recognition of the validity of many traditional claims regarding the value of natural products in health-care (Duraipandiyan and Ignacimuthu, 2007). Medicinal and aromatic plants are gradually attracting a lot of contemporary plant researchers because some human diseases resulting from bacterial antibiotic and fungal antifungal (fungicide) resistances have gained worldwide concern (Cowan, 1999; Ghannoum and Rice, 1999; Kumar *et al.,* 2006).

In many countries, traditional medicine still plays a major role as part of primary health-care, especially in remote rural areas due to availability and cost (Mander and Le Breton, 2006b). Most people living in rural areas rely more on traditional leafy vegetables and herbs that grow in the wild for sustenance and these vegetables may possess some medicinal properties (Jaca and Kambizi, 2011). For minor illnesses such as coughs caused by colds, they prefer using these herbs than consulting allopathic/medical doctors as they still have the perception that western pharmaceuticals and health-care are expensive. By comparison, traditional

medicines are cheap (Fennell *et al.*, 2006) and easily accessible. In South Africa, medicinal plants (locally referred to as *muthi* by the Zulu community and *amayeza* by the Xhosa community) are still widely used in health-care system particularly by the African population (Wiersum *et al.,* 2006). Remarkably, in South Africa medicinal plants are not only used for healing of physical illnesses, but also for protection against misfortunes related to natural and supernatural causes in cultural ceremonies (Cocks and Møller, 2002). It is estimated that 80% of the Black population consult with traditional healers (Jäger and Van Staden, 2000; Fennell *et al.*, 2006). This figure is not surprising due to the diversity in cultures of the Black population and their massive belief in the healing properties or powers of plants.

2.1.2. Commercialisation of medicinal, aromatic plants and traditional medicine in southern Africa

It is interesting to note that in southern Africa, the use of traditional medicine is not only confined to rural, home income groups, but also prevails in urban areas. The trade of MAPs and plant-derived medicine forms part of a multimillion-dollar 'hidden economy' (Cunningham, 1989) and this is mainly as a result of affordability, accessibility and acceptability of traditional medicine over western medicine, and a high rate of unemployment and low level of former education, especially in rural areas (Williams *et al.,* 2000; Dold and Cocks, 2002). The unsustainable use stemming from intense harvesting from wild species is due to the high demand of these MAPs and is therefore a management issue facing conservation agencies (Cunningham, 1997). For example, the threat to *Cassipourea flanaganni* (Schinz) Alston (rare *spp.*) and *Ocotea bullata* (Burch.) Baill. (vulnerable *spp.*) due to medicinal plant overharvesting has been documented by Dold and Cocks (2002), Geldenhuys and Van Wyk (2002) respectively. The effect of overharvesting of *Pelargonium* species in the Eastern Cape is also a major concern (Makunga *et al.,* 2008).

At present, the trade in plant parts and plant-derived medicine is higher than it has ever been. However, the medicinal plant industry in southern Africa remains largely

an informal industry with virtually no official trade statistics (Mander *et al.,* 2006a, 2006c) as volumes of materials traded are difficult to quantify at regional markets which may lead to imprecise national markets (Makunga *et al.,* 2008). The little statistics available from previous studies indicate that up to 700,000 tonnes of plant material is consumed annually with an estimated value of as much as 150 million US dollars per annum (Mander and Le Breton, 2006b); nevertheless, less than half of this revenue is generated through formal market trading. The larger informal market is in KwaZulu-Natal followed by Gauteng (Witwatersrand) (Williams *et al.,* 2007) and involves the trade of non-renewable unprocessed (bulb, rhizome and bark) and semi-processed products (Mander, 1998). Dold and Cocks (2002) conducted a case study of the trade in medicinal plants in four trading regions in the Eastern Cape Province (Port Elizabeth/Uitenhuge, East London/King William's Town, Mthatha and Queenstown) which revealed that a minimum of 166 medicinal plant species were traded in these regions alone providing 525 tonnes of plant material valued at approximately 4 million US dollars annually. It is important to note that regional studies are imperative in documenting trade of medicinal plants as trade differs considerably within regions. The study also revealed that 93% of the species documented were harvested unsustainably, with bark being stripped from trees and underground tubers being extracted from the soil, and 34 species have been prioritized for conservation management.

The markets in small rural areas like the Lowveld (a region with low lying plains situated east of the Drakensberg mountain range) and the Eastern Cape tend to be smaller and more fragmented (Botha *et al.,* 2007). Nevertheless, even though trading in these small rural areas is less, harvesting is on the increase to meet urban demands (Makunga *et al.,* 2008). It is also interesting to note that prices are not the same between and within regions; pricing structures fluctuate between markets and overtime as the cost of plants per unit mass increases or varies depending on a gatherer's access to resources and the proximity of markets of the harvesting sites (Botha *et al.,* 2007). A characteristic of the medicinal plant trade is the flexibility in the nature of the transactions, that is, the selling of plants at negotiable prices and the absence of any contractual relations of production (Williams *et al.,* 2007). This flexibility in trading presents a huge risk on plant diversity as there is now growing

shortages in supply of popular medicinal plant species, as a result there is a trend where there is more escalation than ever of harvesting pressures on traditional supply areas.

With the increased realisation that some wild species are being overexploited and the future demand of medicinal plants is to be met for commercial needs, certain agencies have recommended adoption of policies to promote domestication (reported by the World Health Organisation (WHO), International Union for Conservation of Nature (IUCN), World Wildlife Fund (WWF) in 1993). Most South African conservation agencies have now initiated community-based conservation (CBC) programmes with some traditional healers and, more recently, those involved in trade (Botha *et al.,* 2004). The South African government (Department of Science and Technology, DST) is also providing funding to research councils such as: the Council for Scientific and Industrial Research (CSIR), the Institute for Natural Resources (INR) and the Medical Research Council (MRC) to transfer medicinal plant propagation and value-addition technology for the establishment of small-, micro- and medium-sized enterprises (SMME's) that commercially produce indigenous medicinal plants (DST, 2003). However, there has been a low adoption rate of this concept by traditional healers. This can be explained by various misconceptions that cultivated plants are sometimes inferior in quality when compared with wild gathered specimens (Schippmann *et al.,* 2002). Species that traditional healers grow in their gardens are those for treating common ailments and plants used for protecting houses against lightning. According to Cunningham (1994) in Botswana, traditional healers said that cultivated material was unacceptable, as cultivated plants did not have the 'power' that the material collected from the wild has. Intensive education programmes are still needed to make traditional healers understand the importance of cultivation with intended purpose of conserving wild species of medicinal plants.

2.1.3. Importance of medicinal plants for new product discovery

Medicinal plant usage has evolved to be an important element in the health-care delivery system in both the urban and rural African communities. Tracing back the use of plants as medicine, plant derived medicines initially took the form of teas, tinctures, poultices, powders, and other herbal formulations (Balunas and Kinghorn, 2005). Recipes and methods of application for particular ailments were passed down from generation to generation (Arber, 1986). Today, 38 South African indigenous species out of 3000 medicinal plant species regularly used in traditional medicine have been commercialised to some extent. These are available in the pharmaceutical and cosmeceutical industries and are packaged in the form of teas, tablets, capsules and / or ointments (Van Wyk, 2008). Southern African medicinal plants such as *Perlagonium sidoides* DC., *Sutherlandia frutescens* (L.) R.Br., *Hoodia gordonii* (Masson) Sweet ex Decne., *Lippia javanica* (Burm.f.) Spreng, *Artemisia afra* Jacq. ex Willd., *Aloe ferox* Mill. and others are currently extensively studied and have sparked a lot of interest in crop and product development (Van Wyk, 2008). The role played by these medicinal plants in the provision of novel agents having potential in the treatment and prevention of many diseases such as cancer, leukemia, human immunodeficiency virus or acquired immune deficiency syndrome (HIV/AIDS), malaria and other serious diseases have been on the review.

For example, a triglycoside 12β-tigloyloxy-14β-hydroxypregn-5-en-20-one (designated P57) has been identified as an active appetite suppressant component of *Hoodia gordonii,* a plant which has attracted worldwide attention having about 20 international patent application/registrations and many *Hoodia*-containing commercial preparations in the market (Van Heerden, 2008). The discovery and development of antileukemic agents, vinflunie (a modification of vinblastine) and other essential compounds from *Catharanthus roseus* (L.) provided convincing evidence that plants could be sources of novel products for treatment of various ailments (e.g. cancer) (Baker *et al.,* 1995; Balunas and Kinghorn, 2005). However, the process of developing medicinal plants into new products is complex as it requires a multidisciplinary approach. Fundamental biological knowledge about a candidate species including its phylogeny, taxonomy, chemical variation and

reproductive biology needs to be obtainable. Based on this approach, it takes many years of systematic and concerted effort to select the best candidate plant species for a specific biological activity. Moreover, it is a challenging task in terms of monetary demands (Kamatou, 2006). Despite the diversification in the drug discovery approach from medicinal plants and the time line, natural products from plants and other biological sources remain an undiminished source of new pharmaceuticals.

2.1.4. Problems related to microbial pathogens and approaches to new drug discovery

Microbial pathogens such as fungi, bacteria, parasites and viruses causing infectious diseases still pose a major threat to public health despite tremendous progress in medicine (Cos *et al.,* 2006; Kamatou *et al.,* 2007). Problems posed by these microbial pathogens is particularly pressing in developing countries due to poverty, ignorance, poor sanitation, hunger and malnutrition, unavailability of medicine, and the emergence of wide spread resistance of pathogens to the available drugs (Byarugaba, 2004). To complicate matters even further, bacterial infections contributing most to human and animal diseases in developing countries, are also those in which antimicrobial resistance is most evident (Okeke *et al.,* 1999). *Streptococcus pneumoniae,* for example, is an important pathogen in many community-acquired respiratory infections in the United State and a leading cause of morbidity and mortality worldwide (Appelbaum, 2002). In South Africa alone, resistance to penicillin among South African strains of *S. pneumoniae* increased from 4.9% in 1979 to 14.4% in 1990 (Koornhof *et al.,* 1992).

A new spectrum of human fungal resistance is also on the rise due to increased incidences of cancer, HIV and AIDS, and infected numbers of immunocompromised patients (Arif *et al.,* 2009). Up to 90% of HIV-positive individuals contract fungal infections of which 10 to 20% die as a direct consequence of these infections (Samie *et al.,* 2010). *Candida albicans* and *Cryptococcus neoformans* are the most opportunistic infections in HIV/AIDS patients. Not only humans are facing the

problem of resistance development against fungi, farmers worldwide are experiencing wide losses in maize yield due to fungal pathogens such as *Fusarium spp*. *Fusarium verticilliodes* is now known to be one of the most prevalent seed-borne fungi of maize throughout the world (Kriek *et al.,* 1981). *Fusarium* species are also known to secrete mycotoxins that may be fatal to humans and animals having carcinogenic effects (Velluti *et al*., 2000). The drugs currently available to treat fungal infections have serious drawbacks and the occurrence of fungicide resistance is accompanied by toxic side effects (Arif *et al.,* 2009). Amphotericin B, the broad-spectrum drug was the sole antifungal drug for 30 years but has some disadvantages as it is implicated in nephrotoxicity in patients. Therefore, there is a need to search for alternative control methods that may not present the users with any side effects; medicinal plants emanate as possible candidates for solving this dilemma. Patients with suppressed immune systems also become more susceptible to fungi (such as *Fusarium*) which are not normally associated with human diseases.

Since 1994, there has been a lot of research conducted on South African indigenous medicinal plants with the hope of discovering new bioactives potent against some of these importunate microbial pathogens. Medicinal plants might represent an alternative treatment in non-severe cases of infectious diseases. About 25% of potent drugs including antimalarial, antibacterial and antidiabetic compounds have been purified from medicinal plants (Schmidt *et al.,* 2008). Selection of candidate plants is mostly based on prior knowledge of indigenous people on the usefulness of some medicinal plants. For example, traditional healers claim that some medicinal plants such as *Bixa spp* and *Bidens spp* are more effictive in treating infectious diseases than synthetic antibiotics (Rojas *et al.,* 2006). However, such claims require scientific validation.

2.2. The genus *Salvia*

2.2.1. Botany and geographical distribution of *Salvia runcinata* L.f.

Salvia (commonly known as sage) is a large and polymorphous genus which belongs to the mint family, Lamiaceae (Labitae). The family includes 170 to 200 genera and 3 200 to 5 000 species (Riley, 1963). The genus *Salvia* encompasses about 900 shrublike, plant species with an almost cosmopolitan distribution (Hegde, 1992) of which 26 indigenous species are scattered throughout southern Africa (Codd, 1985; Jäger and Van Staden, 2000) and in addition to these, at least four species such as *Salvia coccinea* Buc'hoz ex Etl., *Salvia officinalis* L., *Salvia reflexa* Hornem., *Salvia sclera* L. and *Salvia tiliifolia* Vahl have been introduced. *Salvia* is widely concentrated in the Mediterranean area, and is rare in alpine or arctic regions (Riley, 1963). It includes several ornamental, culinary and medicinal herbaceous species (Kamatou, 2006).

According to Arnold and De Wet (1993), southern Africa is home to more than 24,000 higher plant taxa and a large proportion of these are endemic in character (refer also to Kamatou *et al.,* 2008). Most of the South African *Salvia* species are confined to the Cape region (Kamatou *et al.,* 2008). *Salvia runcinata* is a very variable species and it is extending from Limpopo Province and Botswana to Northern Cape Province, Free State Province, Eastern and Western Cape Province as far south as the Bredasdorp district but is rare in the former Transkei, KwaZulu-Natal and Lesotho (**Figure 2.1**). It grows in a variety of habitats, but usually on heavy soils, sometimes spreading and is common on disturbed places or overgrazed veld, for example under thorn trees (Codd, 1985).

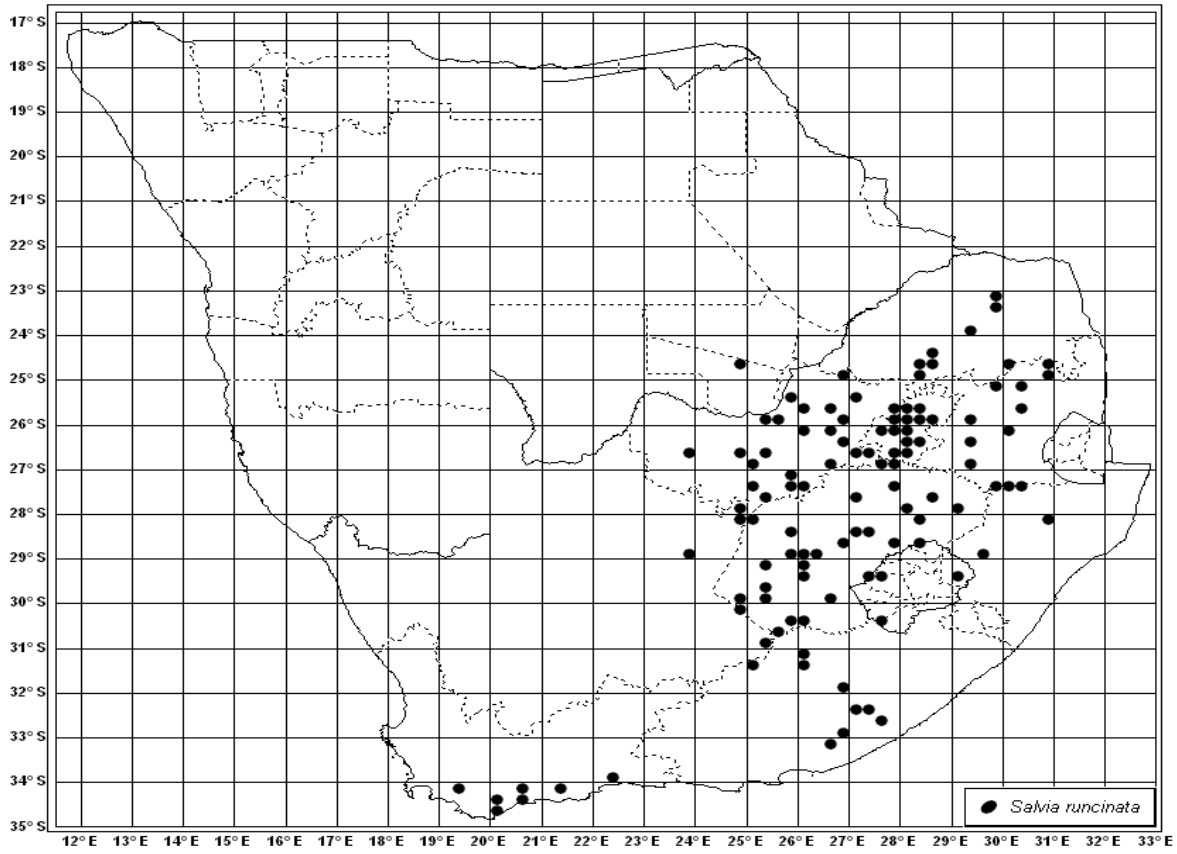


Figure 2.1 The distribution of *Salvia runcinata* in southern Africa (reproduced from South African National Biodiversity Institute (SANBI) National Herbarium Pretoria Computerised Information System (PRECIS).

2.2.2. Morphological description

The mint family has such distinctive features and so it can easily be separated from others (Bhattacharyya and Johri, 1998). *Salvia* species are easily recognised by their quadrangular herbaceous or woody stems and opposite or whorled, simple to pinnately compound pairs of leaves that are usually velvety, glandular and/or hairy on the surface (Riley, 1963; Kamatou *et al.,* 2008). These glands contain volatile oils which make the leaves aromatic (Bhattacharyya and Johri, 1998). The inflorescence is typical of the family and consists of spike or raceme of pairs of dichasial or circinnate cymes or flowers solitary in each axil (Riley, 1963; Bhattacharyya and Johri, 1998). The flowers and stems are key diagnostic characteristics for identification of the genus (Hedge, 1974; Codd, 1985). *Salvia* grows to its optimal

rate in full sun and needs well-drained soil; the roots may rot in waterlogged soil (Kamatou *et al.,* 2008).

The variation of *Salvia runcinata* consists of integrating forms which do not warrant taxonomic recognition. The limits of the species are also far from clear. *Salvia stenophylla* Burch. ex Benth. is probably the closest ally of *Salvia runcinata* (Codd, 1985). This variation can be explained by the genus' ability to readily cross-pollinate forming innumerable hybrids. *S. runcinata* (**Figure 2.2**) is a perennial erect herb that grows from 0.15 to 0.5 meters tall with one to several stems from the taproot or, occasionally, from a creeping rootstock and they are gland dotted (Codd, 1985).

Figure 2.2 (A) A two month old acclimated *in vitro* derived *Salvia runcinata* plant having typical growth and developmental characters, (B) flowers which are similar to those plants that were not *in vitro* propagated.

Salvia runcinata flowers from August to April and the mature calyx is green with a mauve corolla having purple nectar guides. Though morphological differences between *S. runcinata* and *S. stenophylla* are not clearly noticeable, the latter has a

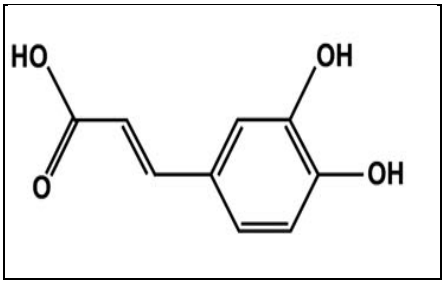
pale blue to mauve corolla with white patches at the center of the upper and lower lips and navy blue nectar guides (Viljoen *et al.,* 2006). Both species are typically aromatic with glandular trichomes that store the essential oils.

2.2.3. Traditional uses and biological activity of *Salvia*

The solvent extracts and essential oils produced from *Salvia* species display a broad range of pharmacological properties, both *in vivo* and *in vitro* (Kamatou, 2006). Biological activities are correlated to the presence of chemical compounds, particularly secondary metabolites. The presence of these compounds may assist in predicting some traditional uses of these medicinal plants (Kamatou *et al.*, 2008). A wide variety of species of the *Salvia* genus show also variable bioactivity. There are, however, many differences in pharmacological actions among these species. *Salvia* is a rich source of polyphenolics, with an excess of 160 polyphenols having been identified, some of which are unique to the genus (Loo and Foo, 2002). The polar phenolic acids constitute the major part of the water-soluble components of *Salvia* decoctions (Loo and Foo, 2002; Kamatou, 2006). The majority of phenolic acids in *Salvia* species are exclusively those of caffeic acid derivatives. According to Loo and Foo (2002), caffeic acid (**Figure 2.3A**) plays a central role in the biochemistry of the Lamiaceae and occurs predominantly in the dimer form as rosmarinic acid (**Figure 2.3B**). In many *Salvia* species, caffeic acid is the building block of a variety of plant metabolites, ranging from simple monomers to multiple condensation products that give rise to a variety of oligomers (Kamatou *et al.*, 2010).

According to Kamatou *et al.* (2010), rosmarinic acid is abundant in *S. runcinata* occurring at a level of 30%. Due to this relatively substantial amount, *Salvia runcinata* may be considered as an alternative commercial source of natural rosmarinic acid. This compound is a natural antioxidant widespread in the families of Lamiaceae and Boraginaceae (Kintzios, 1999) and it contains two phenolic rings, both of which have two ortho-position hydroxyl groups. There is a carbonyl group, an unsaturated double bond and a carboxylic acid between the two phenolic rings (Tepe *et al.,* 2005). This acid has many biological activities such as antiHIV1 action,

antitumor, antihepatitis, antiinflammation activities and also inhibiting blood clots. Additional to this, papers by Tepe *et al.* (2004); Tepe *et al.* (2005); and Tepe *et al.* (2006) concerning the biological activities of *Salvia* species, confirm that this genus has great potential, especially as an antioxidant, for the food and cosmetic industry. *Salvia runcinata* has also been found to have relatively higher phenolic content (Kamatou *et al.*, 2010). A stronger correlation between the total phenolic content (TPC) and the antioxidant activity was observed. This implies that the phenolic compounds are partly, if not totally, responsible for the antioxidant activity.

(A) 

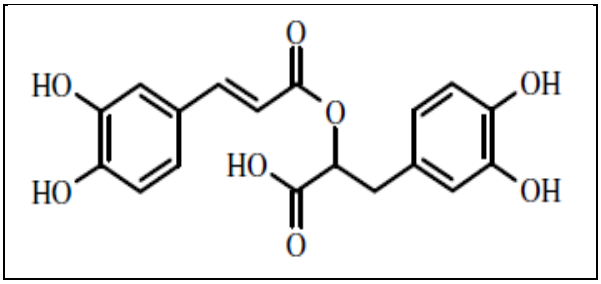
(B) 

Figure 2.3 Chemical structure of (A) caffeic acid and (B) rosmarinic acid adapted from (Tóth *et al.,* 2003).

From the Latin name "*Salvia*" meaning to cure; it is clear that sage has a historical reputation for the promotion of health and treatment of ailments (Cardile *et al.*, 2009). The Latin expression such as: *'Cur moriatur homo cui Salvia crescit in horto*?'-'Why should a man die whilst sage grows in his garden?' epitomises the impact of this sage on that society at the time (Kamatou *et al.*, 2008). *Salvia* is acknowledged worldwide as an important genus because of the beneficial uses of the essential oils produced by the foliage (Ahmed *et al*, 1994) and many *Salvia* species have been used in folk medicines making members of this genus a popular choice for researchers (Kamatou *et al.*, 2005). Until the discovery of antibiotics, *Salvia* was a frequent component of herbal tea mixtures, advocated to patients with tuberculosis to prevent sedation and was found to be an active ingredient in combined plant preparations for the treatment of chronic bronchitis (Cardile *et al.,* 2009). It has also been used as a medication against perspiration, fever, rheumatism, sexual debility and in treating mental and nervous conditions as well as for insecticidal action

(Kamatou *et al.*, 2005). Although today the widespread use of the herb is in flavoring foods, it is still generally used as a household remedy, mainly as an aid in drying up the flow of mother's milk, in reducing saliva secretions in babies, to control night sweats associated with illness and in relieving oral cavity and throat inflammations (Loo and Foo, 1999) suggesting its importance as an antiseptic.

Plants belonging to the genus *Salvia* are known for their many biological activities, such as antibacterial, antioxidant, antitumor, antidiabetic, antimicrobial, anxiolytic, sedative and antiinflammatory activities (Ryu *et al.,* 1997; Kamatou *et al.*, 2008). Because of antimicrobial effects and tannin-based astringent activities of sages, they are used as active ingredients in dental-care preparations. They reduce growth of plaques, gingival inflammation, and have beneficial effects on caries prophylaxis (Baricevic and Bartol, 2000). The Kwena and Tswana community take a decoction of the root of a *Salvia* species in large doses for the treatment of gastric distress caused by a disorder of the liver or gall bladder (biliousness) whilst the Nama take a decoction of *Salvia* species for colds and febrile attacks. Also, they sometimes add it to the bath for other conditions (Watt and Breyer-Brandwijk, 1962).

Specifically, *Salvia runcinata* has been used by different south-eastern African tribes for treating bad sores, herpes and skin lesions, stinging nettle rash, and swellings due to insects or mosquito bites or wasp stings (Watt and Breyer-Brandwijk, 1962). It is used as a decoction, tea or simple lotion. Sometimes milk would be used to steep the sage in with excellent results (Dweck, 2000). The southern Sotho burn *Salvia runcinata* leaves in a hut to disinfect it after sickness and to drive away insects. They also mix the leaves of the plant with their tobacco. A decoction of the root, stem and leaf parts was used by the Europeans for the relief of urticaria (Watt and Breyer-Brandwijk, 1962). The Zulu community uses a paste of crushed leaves as a purgative for infants, while the Xhosa community administers extracts of the leaves to newly born babies (Kamatou *et al.*, 2008). Southern Sotho women, in order to ward off miscarriage and to relieve menstrual pain, take a decoction of *Salvia runcinata* together with *Sopubia cana* Harv. (Watt and Breyer-Brandwijk, 1962).

2.2.4. Conservation status and sustainable use

Natural plant products are usually insufficient or difficult to obtain, hence, substitute methods of desired resources are dimmed necessary (Shaik *et al.*, 2010). In South Africa, conservation has been a bastion of the older paradigm that was less people friendly especially with regards to the poor. With this paradigm, conservation represented the values and principles of the Apartheid approach and discourse (Fakir, 2001), and so, the conservation status of *Salvia* in South Africa and many other ethnobotanicals still remain unknown (Kamatou *et al.*, 2008). South African endemic plants, among other medicinal plants found in the country are likely to face increasing threats as there is an enormous demand as they are heavily utilized to manufacture low-cost phytopharmaceutical drugs (Shaik *et al.*, 2010) and as ethnoherbal products to treat a variety of ailments (Makunga and Van Staden, 2008). This could possibly result in the loss of natural populations to the point of extinction as well as lowered genetic diversity of species; reducing gene flow (Shaik *et al.*, 2010). In general, there is limitation in the cultivation of these medicinal plants in people's gardens, and due to this, most of these plants with medicinal properties are gathered from the wild.

The parliament of the Republic of South Africa enacted laws such as the Biodiversity Act (Act No. 10, 2004) to provide for the management and the conservation of South Africa's biodiversity, which includes the sustainable use of indigenous biological resources such as medicinal plants. Nevertheless the tradition of indiscriminate harvesting of natural population perpetuates. Adding to the threat of unsustainable harvesting or overexploitation is the rising number of infrastructure development in suburban areas such as along the Western Cape coast and, the growing number of alien/invasive species in vulnerable habitats with large endemic flora such as in the fynbos biome (Makunga *et al.,* 2008). These threats are most likely to have a negative impact on the natural succession of endemic plants, remarkably decreasing plant populations from the fynbos biome (Makunga and Van Staden, 2008). There has been considerable effort in the use of plant cell cultures for *ex situ* conservation as an alternative for the production of pharmaceutically active compounds unique to plants to assist with biodiversity conservation (Kamatou *et al.,* 2008).

2.3. Biotechnological applications on *Salvia* species

2.3.1. Recombinant DNA technology

Despite several great efforts employed by the chemical industry to mimic and synthesise specific plant secondary metabolites, little success has been achieved and in general, plants still remain the major supply of many essential medicinal compounds (Wink, 1990). Plants are known to contain more than 100,000 natural organic constituents, many of which are economically important to Man as a source of pharmaceuticals, flavours, fragrances, insecticides, dyes and food additives for example (Cseke *et al*, 2006). With respect to the pharmaceutical interest of these compounds, as many as more than 60% of successful drugs are of natural origin (Cragg *et al.,* 1997). Nevertheless, it is known that many of these metabolites are produced in low amounts in their native host, and their synthesis depends on the physiological and development stages of the plant during its lifespan. Scientific advances made by a mixture of biochemistry, molecular biology and genetics (i.e. recombinant DNA) on studies of biosynthetic pathways of target secondary metabolites have allowed the identification, function determination, characterisation, cloning and expression of many genes. These have positively shed some light into the intricacy of secondary metabolism pathways and their regulation, and have permitted redirection of networks to accumulate tailored metabolites. In simpler terms, the primary objective of recombinant DNA technology is the identification and isolation of genes with a goal of creating useful products for a commercial pipeline (Glick and Pasternak, 2003).

The use of recombinant DNA technology still holds the promise of enhancing plant availability and growth, the ability to enhance the production of the essential secondary metabolites and grow more and better food with increased nutritional value despite negative public perception. Genetic engineering has allowed the production of plants with an altered content of secondary metabolites (Floryanowicz-Czekalska and Wysokińska, 2000). This technology comprises of a battery of experimental procedures that are used for inserting DNA molecules from one

organism into a vector, often a bacterial plasmid, and perpetuating the insert DNA-vector DNA combination in a host cell. Two major categories of enzymes are important tools in the isolation of DNA and preparation of recombinant DNA (Mullis, 1990). These are the type II restriction endonucleases or restriction enzymes that cleave DNA molecules reproducibly into fragments of discrete sizes (Glick and Pasternak, 2003). They bind to specific sequences within a DNA molecule and symmetrically cut phosphodiester bonds of each strand at the recognition site. The other types of enzymes such as the DNA ligase and DNA polymerase are also important for cloning genes.

2.3.2. *Agrobacterium*-mediated transformation

Plants remain a major source of pharmaceutical and fine chemicals. Despite considerable efforts, only a few commercial processes have been achieved using cell cultures. The major constraint with cell cultures is their genetic instability and they tend to produce low yields of secondary metabolites (Giri and Narasu, 2000). In addition to the use of the recombinant DNA technology discussed above, direct and indirect gene transfer methods have been used in medicinal plants (Nigro *et al.,* 2004). One of the methods that have been used since the 1980's as a route for enhancing secondary metabolite production involves using the natural vector system *Agrobacterium rhizogenes*, which is the causative agent for the hairy root disease in plants.

Genetically transformed hairy roots obtained by infection of plants with *A. rhizogenes*, a Gram-negative soil borne bacterium, still offers a promising system for secondary metabolite production, phytochemicals (Shanks and Morgan, 1999), monoclonal antibody production (Wongsamuth and Doran, 1997) and phytoremediation (Nedelkoska and Doran, 2000). Traditional hairy root cultures are very useful, as they grow rapidly, are genetically stable and capable of synthesizing metabolites found in the biological roots and other plant organs (referred to as green hairy roots), in abundance (Królicka *et al.*, 2001). Moreover, transformed roots are able to regenerate whole viable plants and maintain their genetic stability after

subculturing and plant regeneration. A root is referred to as a 'transformed root' when it harbors a 'foreign' transfer-DNA (T-DNA) gene segment from *Agrobacterium rhizogenes's* root-inducing (Ri) plasmid in its plant cell.

2.3.3. Structure of T-DNA from *Agrobacterium rhizogenes* and the molecular basis of *Agrobacterium*-mediated transformation

The virulent strains of the phytopathogen *A. rhizogenes* contain the root-inducing (Ri) plasmid which possess three different regions important for gene transfer (Chandra, 2012): **1.** the T-DNA which gets transferred and integrated into the plant genome, **2.** the border sequence of the T-DNA and **3.** the virulence area (*vir*) which is responsible for the transfer of the T-DNA (Okasman-Caldenty and Hiltunen, 1996) (**Figure 2.4**).

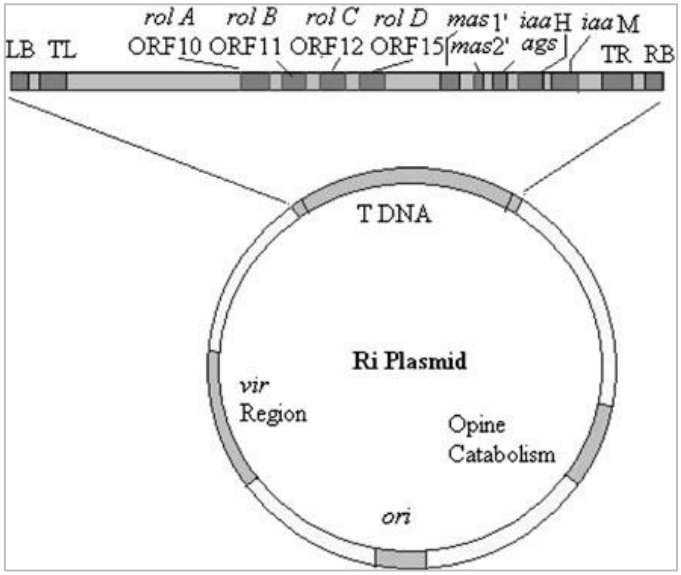


Figure 2.4 Schematic representation of the root-inducing (Ri) plasmid of *A. rhizogenes* (adapted from Chandra, 2012).

The *A. rhizogenes* strains are divided into four classes: agropine, cucumopine, mannopine or mikimopine based on the type of opine they catabolise (Huffman *et al.,* 1987; Sevón and Oksman-Caldentey, 2002; Ramogola *et al.,* 2009). Opines are a group of compounds that serve as specific growth substrates for the pathogenic

agent (*A. rhizogenes*) (Petit *et al.,* 1983). This means that this group of compounds creates a favorable chemical environment for growth and propagation of the pathogenic bacterium thus mediating parasitism. The agropine Ri plasmid T-DNA has two sub-fragments: the left T-DNA (T_L-DNA) and right T-DNA (T_R-DNA) termed ''split'' T-DNA (Veena and Taylor, 2007). Each of the T-DNA fragment spans an 8-20 kb region, and they are separated from each other by at least 15-18 kb of non-integrated plasmid DNA (Sevón and Oksman-Caldentey, 2002) (**Figure 2.5**). The T_R-DNA is not essential for hairy root formation but has been shown to contain genes homologous to auxin biosynthesis (*tms*$_1$ and *tms*$_2$) and genes encoding synthesis for agropine (*ags*) (Ramogola *et al.,* 2009).

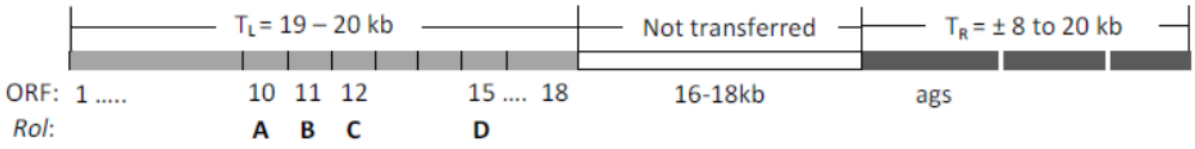


Figure 2.5 A segment of the *A. rhizogenes* agropine Ri plasmid (constituted using information from Tiwari *et al.,* 2007).

The genes of the Ri T_L-DNA direct the synthesis of a substance that recruits the cells to differentiate into roots under the influence of endogenous auxin synthesis (see Section 2.3.4.) (Giri and Nasaru, 2000). The site of insertion of the T-DNA construct appears to be largely random and multiple insertions may occur in the same nucleus. Sometimes the T-DNA construct will insert into a region of a chromosome that is heterochromatic and contains no functional genes (Gelvin, 2000). This means, it is possible for the T-DNA to go into a chromosomal region that does not provide the correct chromosomal architecture for gene expression. The whole transference process of the T-DNA from the bacteria to the host plant genome is mediated by the virulence genes that form the *vir* region of the Ri plasmid or tumour inducing (Ti) plasmid (Huffman *et al.,* 1984), and *chv* genes found on bacterial chromosome (Giri and Nasaru, 2000). The *vir* genes do not enter the plant cell but, together with the chromosomal DNA (two loci) facilitate the transfer of the T-DNA. Transcription of the *vir* region is induced by acetosyringone and to induce higher levels of *vir* gene

expression, various other sugars act synergistically with acetosyringone (Vanhala *et al.,* 1995). The bacterium is attracted through chemotaxis and attaches to wounded plant cells (**Figure 2.6**) (Giri and Nasaru, 2000). The adventitious roots start proliferating abundantly at the infection site subsequent to integration and the expression of the genes that confer the hairy root syndrome.

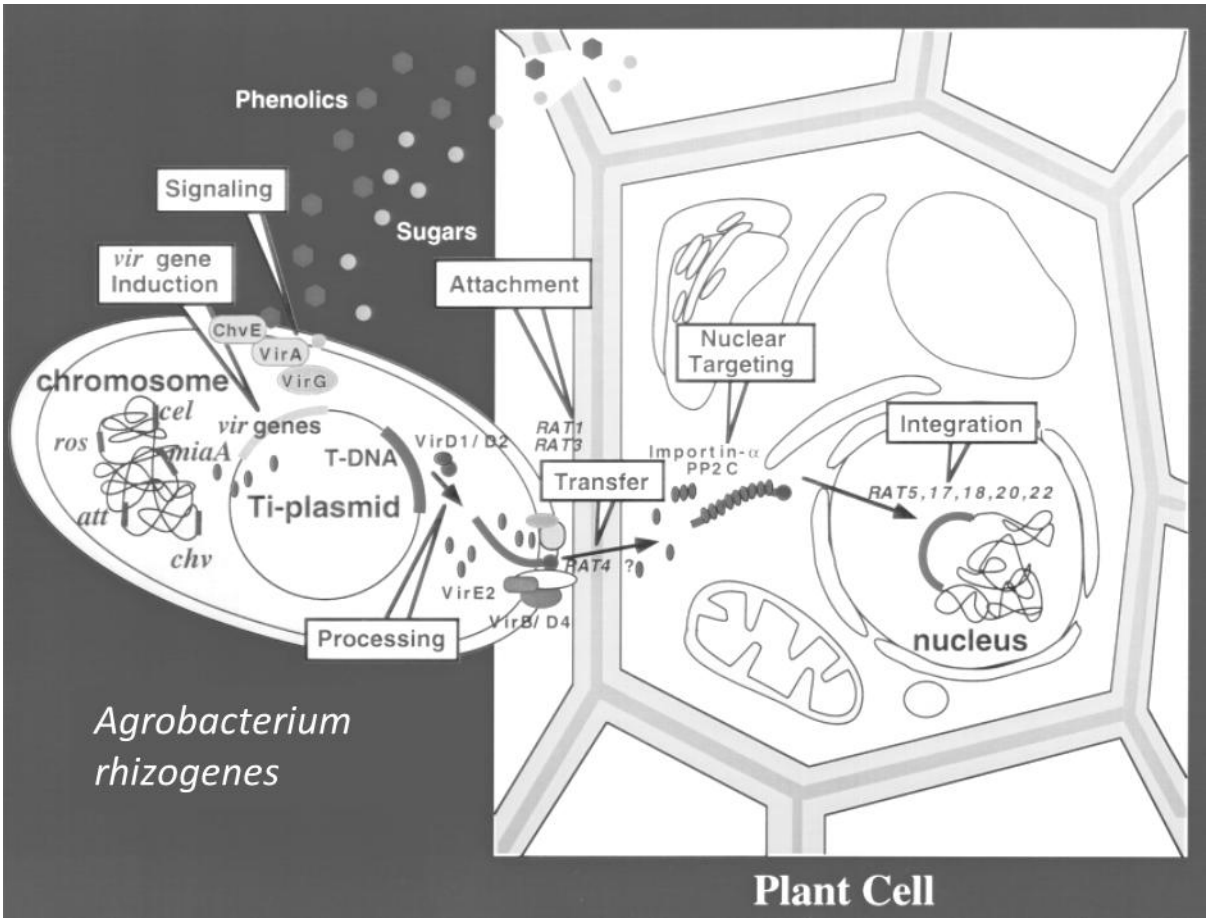


Figure 2.6 Schematic diagram illustrating the mechanism of the *Agrobacterium*-plant cell interaction. Critical steps that occur to or within the bacterium (chemical signaling, *vir* gene induction, and T-DNA processing) and within the plant cell (bacterial attachment, T-DNA transfer, nuclear targeting, and T-DNA integration) are highlighted along with genes, and or proteins known to mediate these events (adapted from Gelvin, 2000).

2.3.4. Genes of the Ri T_L-DNA conferring the hairy root syndrome: A review of the *rol* genes and their functions

As previously noted, mutation analysis of the T_L-DNA has led to the identification of at least four genetic loci, designated locus *rol* A, *rol* B, *rol* C, and *rol* D, which affect hairy root induction (White *et al.,* 1985). The complete nucleotide sequence of the T_L-region derived from clone banks of pRiA4 and pRiHRI revealed the presence of 18-open-reading frames (ORFs) (Slightom *et al*., 1986), 4 of which, ORFs 10, 11, 12 and 15, respectively, correspond to the *rol* A, *rol* B, *rol* C, and *rol* D loci (**Figure 2.5**) (Slightom *et al.,* 1986; Sevón and Oksman-Caldentey, 2002). Transformation work done in tobacco more than 25 years back revealed that a set of only a few genes of pRiA4 T_L-DNA, namely *rol* A, *rol* B, and *rol* C play the most important role in hairy root formation (Cardarelli *et al.,* 1987). Of particular interest, plants containing the ORF11 (*rol* B) locus show the typical hairy root phenotype (Cardarelli *et al.,* 1987) and when this T_L-DNA locus was inactivated on *Kalanchoe daigremontiana* leaves, this led to avirulence of the agropine-type *A. rhizogenes* (White *et al.,* 1985). Therefore, *rol* B seems to be the most crucial in the differentiation process of transformed cells, while *rol* A and *rol* C provide with accessory functions (Sevón and Oksman-Caldentey, 2002) and are not essential for inducing transgenic root initials.

Genetic transformation mediated by *Agrobacterium rhizogenes* is however affected by the explant genotype, structure and developmental stage (Chriqui *et al.,* 1996), chemical and physical factors, bacterial strains and signal molecules (Akramian *et al.,* 2008). Chriqui and his colleagues (1991) found out that only juvenile seedlings of both *Eucalyptus gunnii* and *E. globulus* were able to respond to inoculations through a rooting response but mature plants never displayed any symptoms. In a study by Akramian and his colleagues (2008), five *A. rhizogenes* strains used in their experiments demonstrated significant difference in virulence. This suggests that transformation experiments, like any other experiments, require optimization steps using different strains, different explants and different medium conditions to get to the desired result.

Since the 1980's, technology based on *A. rhizogenes* has been investigated for secondary metabolite production and the effect of the different *rol* genes in plants. Cardarelli and his colleagues (1987) cloned various segments of the T_L-DNA of the agropine-type Ri plasmid pRi1885 encompassing single and groups of ORFs in the Ti plasmid-derived binary vector system Bin 19. Interestingly segments encompassing the single ORF11 corresponding to the *rol* B locus resulted in transformed *Nicotiana tabacum* plants, some of which showed typical hairy root phenotypes such as wrinkled leaf morphology and the ability of leaf explants to differentiate roots in hormone-free culture medium. Colling *et al.* (2010) transformed *Perlagonium sidoides* with the *rol* genes and established highly regenerative hairy roots. These roots produced commercially important coumarins, flavonoids and phenolic acids in significantly higher amounts. Ramogola *et al.* (2008) also successfully established several root clones of *Salvia africana-lutea* L. using conventional *A. rhizogenes* transformation and the clones were subsequently tested for their pharmacological actions. It is interesting to note that metabolite profiling of the transgenic root cultures showed fascinating changes in the chemical footprint of *S. africana-lutea.*

Therefore tissue culture and *Agrobacterium*-mediated transformation techniques have potential as *in vitro* conservation systems and may be useful for the production of new subsets of secondary metabolites in important medicinal South African indigenous plants or at least lead to higher levels of secondary metabolites generally associated with particular species.

2.4. Motivation and Rationale

Products from the genus *Salvia* have a huge potential for commercialisation due to their proven abilities to cure a wide variety of diseases. However, there has been limited biotechnological application on *Salvia* species indigenous to South Africa. In terms of micropropagation, in an attempt to conserve South African native *Salvia* species, there are only 3 species of this genus with established micropropagation protocols (Huang and Van Staden, 2002; Makunga and Van Staden, 2008; Musarurwa *et al.,* 2010). According to our knowledge, a micropropagation protocol of *Salvia runcinata* is non-existent. Viljoen and his colleagues (2008) have conducted studies assessing the volatiles of wild growing *Salvia runcinata* plants from different environments. This current research adds value to *S. runcinata* research that is already in the public domain by applying biotechnology tools that potentially alter the metabolome. The expectation of up-regulating the synthesis of compounds or bioactives such as epi-α-bisabolol was hoped to lead to enhancing the biological activity of the plant.

2.5. Aims and Objectives

This project thus aimed at enhancing the secondary metabolite production of *Salvia runcinata* through micropropagation and transformation using two conventional *Agrobacterium rhizogenes* strains. This was followed by testing tissue cultured and glasshouse plants plus hairy roots for their pharmacological activities. The objectives of the study were therefore:

- to develop an efficient tissue culture system for *Salvia runcinata* as there was none available;
- to transform *S. runcinata* using *Agrobacterium rhizogenes* strains A4T and LBA 9402 by testing different explants;
- to analyse the phytochemistry of transgenic and non-transgenic *S. runcinata* plants using GC-MS profiling;

- to investigate antibacterial and antifungal activities of transgenic and non-transgenic material using a microdilution bioassay.

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Chapter 3

Seed germination and micropropagation of *Salvia runcinata* L.f. through regeneration of adventitious shoots

3.1. Introduction

Throughout the world there is a strong relationship between the history of the people and traditional use of plants (Lewis and Elvin-Lewis, 1995; Agosta, 1997; Cunningham, 2001). Fossil records date the use of plants by men as medicines at least to the Middle Paleolithic age, some 60 000 years ago (Fabricant and Farnsworth, 2001) and today, plants still continue to provide mankind with new remedies. According to the World Health Organisation (WHO), a large proportion of the population in many developing countries (approximated to 80%) still relies heavily on medicinal plants to meet primary health-care needs. Focussing on southern Africa, which is endowed with a rich floral diversity that is largely endemic in character (Goldblatt, 1978; McGaw *et al.*, 2005), a majority of the people consult with more than 100 000 practising traditional healers (Mander, 1998) who derive their medicinal preparations from more than 1020 plant and 150 animal species (Mander, 2008; Koduru *et al.*, 2007).

Today, the South African population is nearing 51 million and growing at a rate of 1.1 % (UNICEF, 2006). This growing population in South Africa in the past years has culminated in; (among other things) higher levels of urbanisation and the increasing demands on traditional health-care which in turn have resulted in a scenario where the supply of medicinal plants from the wild can no longer meet the needs (Taylor *et al.*, 2001a, 2001b). There is still a continuous problem of unsustainable harvesting of wild populations and climate changes; such that some of South Africa's indigenous plants are facing extinction. To mention a few of threatened southern African indigenous species; *Siphonochilus aethiopicus* (Schweif.) B.L. Burt (Zingiberaceae), *Ocotea bullata* (Burch.) Baill. (Lauraceae) and *Warburgia salutaris* (Bertol.f.) Chiov.

(Canellaceae) are almost extinct in the wild. *W. salutaris* only grows in six protected areas in South Africa with its bark currently being imported from Zimbabwe, Mozambique and Swaziland (Zschocke *et al.*, 2000). This is affecting the natural wealth of the continent, the southern African region in particular and future generations will not be able to indulge on the natural beauty and rich resources of the country.

It is clear that there is a great deal of pressure exerted on the plants from the wild including medicinal plants. With this realisation of the depletion in wild type species of medicinal plants, in an attempt to save the destruction of the ecosystem, there has been a need for urgent corrective and protective measures. A way of producing more plants that can cater for the needs of traditional plant users is of necessity (Afolayan and Adebola, 2004) and on the other hand, making sure that the plants are of good quality and can be exploited in the pharmaceutical industry is important. Techniques like *in vitro* microplant culture that have been developed, have provided ways to ease the pressure from wild sources through rapid propagation of a large number of uniform plants while maintaining their genotypes and quality (Arikat *et al.,* 2004; Matu *et al.*, 2006). Plant tissue culture has therefore indisputably rescued many species from extinction (Moyo *et al.*, 2011).

The first micropropagation protocol for a *Salvia species*: *Salvia officinalis* L.*,* was reported in 1990 by Olszowska and Furmanowa. Huang and Van Staden (2002), Makunga and Van Staden (2008), Musarurwa *et al.* (2010) have developed efficient micropropagation techniques for three African *Salvia* species, *Salvia chamelaeagnea* P.J.Bergius, *S. africana-lutea* L. and *S. stenophylla* Burch. ex Benth. respectively. These authors aimed at generating a continuous supply of large quantities of these medicinally valuable *Salvia* species that are endemic to southern Africa. However, these are the only three *Salvia* species with established micropropagation protocols out of about 26 species that are indigenous to southern Africa. To the best of our knowledge, there is no literature documented on the *in vitro* germination and micropropagation of *Salvia runcinata.*

Salvia runcinata has been shown to have high abundance of epi-α-bisabolol which is a compound important in skin products having antiinflammatory activities (Kamatou *et al.*, 2005). Due to this growing economic value, the demand for the plant is anticipated to increase as it may be required in the cosmetic industry. With the idea of alleviating pressure from wild harvesting of *Salvia runcinata*, the present study was undertaken as a pre-emptive measure with the objective of developing a viable *in vitro* culture technique of germination and micropropagation of *S. runcinata* as an *in vitro* conservation strategy. In addition, the present study was also undertaken to establish the effects of tissue culture on the bioactivity and chemistry of *S. runcinata*.

3.2. Materials and Methods

3.2.1. Seed germination studies

Salvia runcinata (L.f) seeds were purchased from Silverhill Seeds (Cape Town, South Africa) in May 2010 and were stored at room temperature in the dark prior to germination experiments. Annually, all seeds are collected seasonally from the wild populations growing around the Grahamstown area in the Eastern Cape Province by Rod and Rachel Saunders (http://www.silverhillseeds.co.za/collecting.asp). Smoke and a combination of smoke with scarification (detailed below) were used as treatments in this study. The experiments were set up in a completely randomised design and therefore each treatment had 10 Petri dishes (each Petri dish representing a block) and there were 10 seeds (replicates) per block. For the smoke treatment, the seeds were surface-decontaminated in 3.5% (w/v) NaOCl for 20 min and placed onto a medium with 10^{-5} (v/v) concentrated filter-sterile (0.2 µM) smoke solution. The smoke solution was purchased from Kirstenbosch Botanical Gardens (Western Cape, South Africa). This is generally prepared using the method of Baxter *et al.* (1994) from *Themeda triandra* Forssk and sold as a 10^{-5} (v/v) concentrated filter-sterile solution. The sterilised seeds were then washed three times (5 min) with sterile distilled water prior to placing onto half-strength Murashige and Skoog (½ MS) (1962) medium (0.1 g l^{-1} myo-inositol; 0.2 µM smoke solution and 3% (w/v) sucrose solidified with 9 g l^{-1} agar, pH adjusted to 5.8 with 1 M NaOH or 1 M HCl). The MS

salts were prepared by Highveld Biologicals (Pretoria, South Africa) and for each litre, the manufacturer recommended using 4.4 g of the powder for a full complement of macro- and micronutrients including vitamins (**Appendix A**[1]). Prior to surface-decontamination, an acid treatment, 25% (w/v) H_2SO_4, was used for scarification and seeds were exposed for 1 min, 3 min or 6 min to the chemical scarifying agent. The scarified seeds were then placed on smoked ½ MS medium to determine the combinatorial effects of scarification and smoke on germination. As a control, untreated seeds were surface-decontaminated prior to placing onto untreated ½ MS medium. All media were autoclaved at 122 kPa and 120 °C for 20 min. Once cool, 25 ml of the medium was poured into Petri dishes (100 cm X 2 cm from BD Falcon™). Laminar flow conditions were used for all experimental procedures. The Petri dishes were sealed with laboratory film (Parafilm ‘‘M’’ American National Can™, USA) and were transferred to a dark growth room and a 23±2 °C lit growth room with 16/8-h light illumination (50 µmol $m^{-2}s^{-1}$ photosynthetic photon flux density [PPFD]) to induce germination. The light was provided by ‘cool-white’ fluorescent tubes (L75W/20X Osram, USA; code number F96T12). After 24 h, the number of germinating seeds was recorded and thereafter, the seeds were monitored every two days for germination for a period of 31 days. Nevertheless, seeds in the dark room did not germinate over the period of 31 days and therefore this dark room experiment was discarded. Seeds were regarded as germinating once a radicle of 0.5 - 1 cm was noticeable.

3.2.2. Effect of IAA and BA

All germinated seedlings were transferred once more to ½-strength plant growth regulator (PGR)-free MS medium, allowed to develop for a further two months prior to the use of PGRs to assist with shoot multiplication, shoot elongation and root production. Primary *in vitro* plants were aseptically cut so as to generate three types of explants viz. nodal sections (1 cm), leaves (1 x 1 cm) and stems (2 cm). Nodal sections had two axillary buds on opposite side of the stem section. All explants were placed on a shoot multiplication medium containing PGRs added individually or in combination. The nutrient medium consisted of ½ MS medium supplemented with 3% (w/v) sucrose, 100 mg l^{-1} myo-inositol, auxin: cytokinin combinations (µM); IAA:

BA (5.7: 4.43; 5.7: 8.9; 11.4: 4.43; 11.4: 8.9), auxin (µM); IAA: (5.7 or 11.4), cytokinin (µM): BA (4.43 or 8.9), solidified with 9 g l^{-1} agar, pH 5.8. The control experiment consisted of ½ MS medium without PGR supplementation. Cultures were transferred to a 23±2 °C lit growth room with 16/8-h light illumination (50 µmol $m^{-2}s^{-1}$ PPFD).

To reiterate, experiments were set up in a randomised design. Four explants were placed per Petri dish with a volume of 25 ml solid medium sealed with laboratory film. For each PGR treatment, eight Petri dishes were used (N = 32). Each replicate was represented by a single explant. Data were collected after one month in culture and several *in vitro* growth and developmental parameters were assessed including the shoot number, shoot weight, root number and callus formation.

3.2.3. Continuous culture, rooting and acclimatisation

All microshoots produced in culture were pooled together irrespective of the PGR conditions used prior to subculture and a continuous culture system was set up on PGR-free medium. After four weeks, plants were subcultured onto fresh medium for two cycles before the first batch of rooted plants (November 2010) were removed from the tissue culture growth room and transferred out of culture for their continued development in the glasshouse. Routinely, plants of *S. runcinata* were maintained as stocks through four-weekly subculture periods. Following two months of growth post rooting with four weeks subcultures, twenty *in vitro* rooted plantlets were washed free of agar using distilled water to minimize pathogen attack (Matu *et al.*, 2006) prior to their transfer to 20 cm pots (Calibre Plastic Pty Ltd) with 1:1 (v/v) mix of vermiculite and soil (Double Grow, Durbanville). Potted plants were housed in a thermostatically controlled glasshouse; controlled from 18 to 30 °C (minimum to maximum temperatures) at Stellenbosch University (Botany and Zoology Department) with natural summer day light and out-door temperatures ranging from 8 to 19 °C (minimum) to 18-30°C (maximum) during November (www.weatherunderground.com). The photosynthetic active radiation (PAR) in the glasshouse ranged from 540 to 810 µmol m^{-2} s^{-1} at midday. The pots were covered with plastic bags to maintain a high relative humidity (about 90%) and they were then

gradually perforated at the corners after seven days to reduce the humidity to about 70%. The plastic was completely removed after two weeks. The plants were watered (drenched) once every fortnight with distilled water by hand. The acclimatisation percentage was recorded eight weeks after the transfer of rooted plants to the soil.

3.3. Data and statistical analysis

Statistical analyses were performed using STATISTICA data analysis software system version 10 (StatSoft, Inc, Tulsa, USA). Data were subjected to analysis of variance (ANOVA). First, the data were checked for normality and the Kruskal-Wallis ANOVA was then applied. The comparisons between the mean values of treatments were made by the least significant difference (LSD) test calculated at the confidence level of 95%. All percentage data were arcsine transformed before ANOVA. The degree of the significance amongst different treatments was determined by Fisher LSD post-hoc analysis. Factorial ANOVA was used to determine interactions between the control and the treatments, where appropriate.

3.4. Results and discussion

3.4.1. Germination studies

Seed based multiplication is one of the most effective, realistic and convenient means of propagation for most species (Makunga and Van Staden, 2008). In this study, the efficacy of smoke and scarification treatments for the improvement of germination was tested as an *in vitro* tool to fast-track germination. *Salvia* species often exhibit low germination rates and dormancy, displaying erratic germination patterns (Makunga and Van Staden, 2008) making it difficult to coordinate culture induction experiments from a tissue culture perspective so as to ensure that plants from one seed batch are of a similar age. Smoke and aqueous smoke extracts enhance both seed germination and seedling vigour in a wide variety of plants

(Brown and Van Staden, 1997) which is useful for both agriculture and horticultural purposes. Other studies have indicated that smoke affects endogenous GA synthesis and the ABA content (Krock *et al.*, 2002; Schwachtje and Baldwin, 2004).

Although not reported here, seeds placed in the dark did not germinate; an indication of the importance of light in inducing germination in *Salvia runcinata*. Irrespective of the treatment used to break dormancy, the germination percentage was high as compared to the controls which had poor germination rates (**Figure 3.1**) but seeds which were exposed to smoke and those that were treated with a combination of smoke and H_2SO_4 germinated at significantly higher frequencies than the control ($P \leq 0.05$) (**Figure 3.2**). Nevertheless, there was no significant difference regarding to whether smoke was used individually or, in combination with the scarifying acid. This may imply that germination is dependent on endogenous controls rather than being controlled by the presence of the testa. A wide range of plant species respond readily to smoke as a germination cue as discussed by Light *et al.* (2009), Chiwocha *et al.* (2009) and others. In this study, the highest germination rate of 88% was achieved when seeds were incubated for 3 min in H_2SO_4 and treated with smoke (**Figure 3.1** and **3.3A**). Similarly, Kulkarni *et al.* (2007) recorded high germination frequencies for *Dioscorea dregeana* which was stimulated by smoke-water and by butenolide (one of the active ingredients in smoke) under constant dark conditions.

A butenolide-type compound (3-methyl-2H-furo[2,3-c]pyran-2-one), now also referred to as karrikin 1 (kar_1) was identified as the major active compound in smoke which is responsible for smoke-induced germination in plants. Mechanisms associated with this chemical and others which form a complex of synergistic- and antagonistic-acting molecules regulating smoke-enhanced responses in plants remain poorly resolved at the molecular level (Soós *et al.*, 2010). Apart from increasing the number of germinating seeds, smoke and scarification (3 min) correlated to the exponential (log phase) frequencies regarding the germination rates over time, with this being highly significant ($P=0.000$) in comparison to untreated seeds (**Table 3.1**). First time appearance of the radicle for most of the seed lot was recorded from day 7 until day 22 for smoke and H_2SO_4 treated seeds (3 min) (**Figure**

3.1). Those seeds that were exposed to longer scarification periods (6 min) had lowered germination but this was still superior to controls (*P*=0.003741) (**Table 3.1**).

Although speculative, these data suggest that intense scarification may remove more of the seed coat making the seeds highly susceptible to higher concentrations of the smoke. As smoke-water is composed of a 'cocktail' of compounds and acts against a wide concentration range, supra-optimal levels are known to become inhibitory, maintaining dormancy and reducing the germination potential even under favourable environmental conditions, as some of the smoke-water chemicals are phytotoxic (Soós *et al.,* 2010).

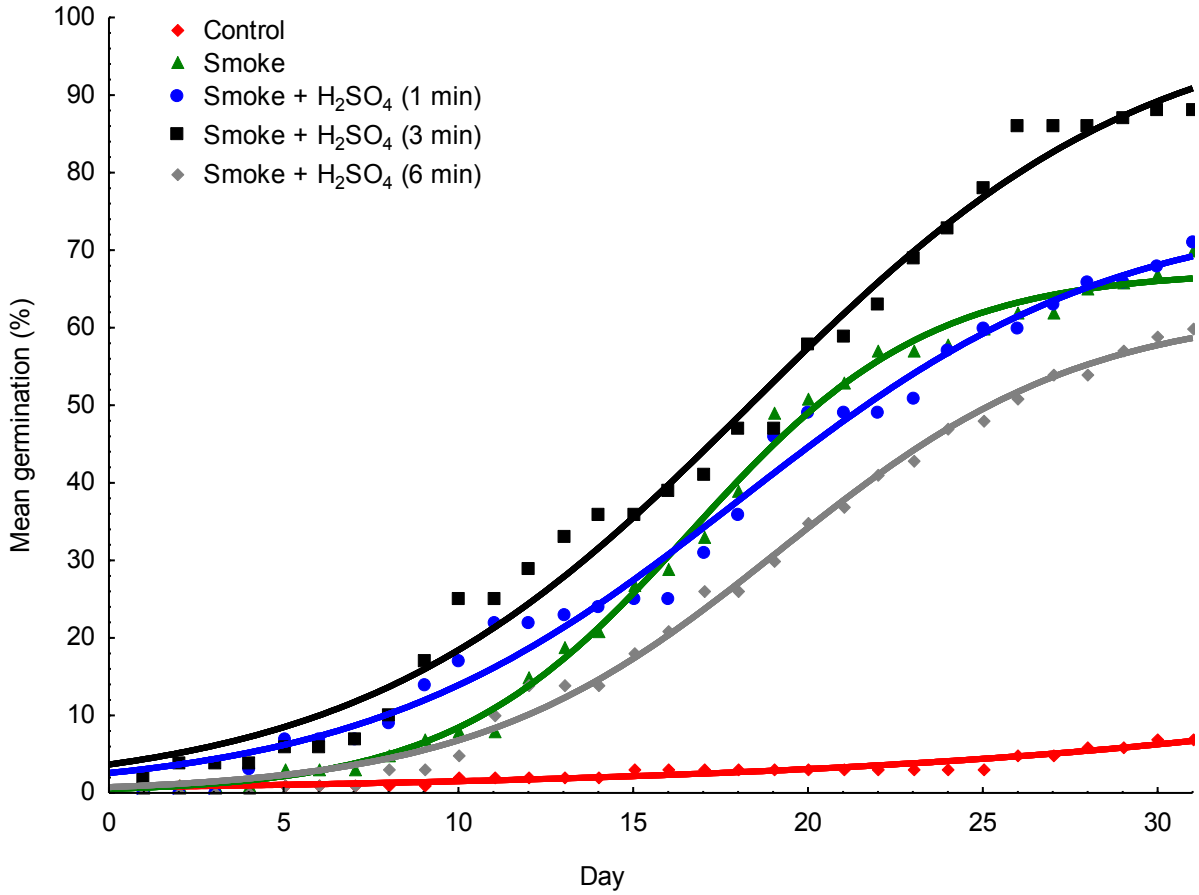

Figure 3.1 Seed germination (%) of *Salvia runcinata* over 31 days as affected by different treatments. The seeds were treated with smoke and a combination of smoke with H_2SO_4. Data were analysed using a repeated measures ANOVA at the 95% confidence interval.

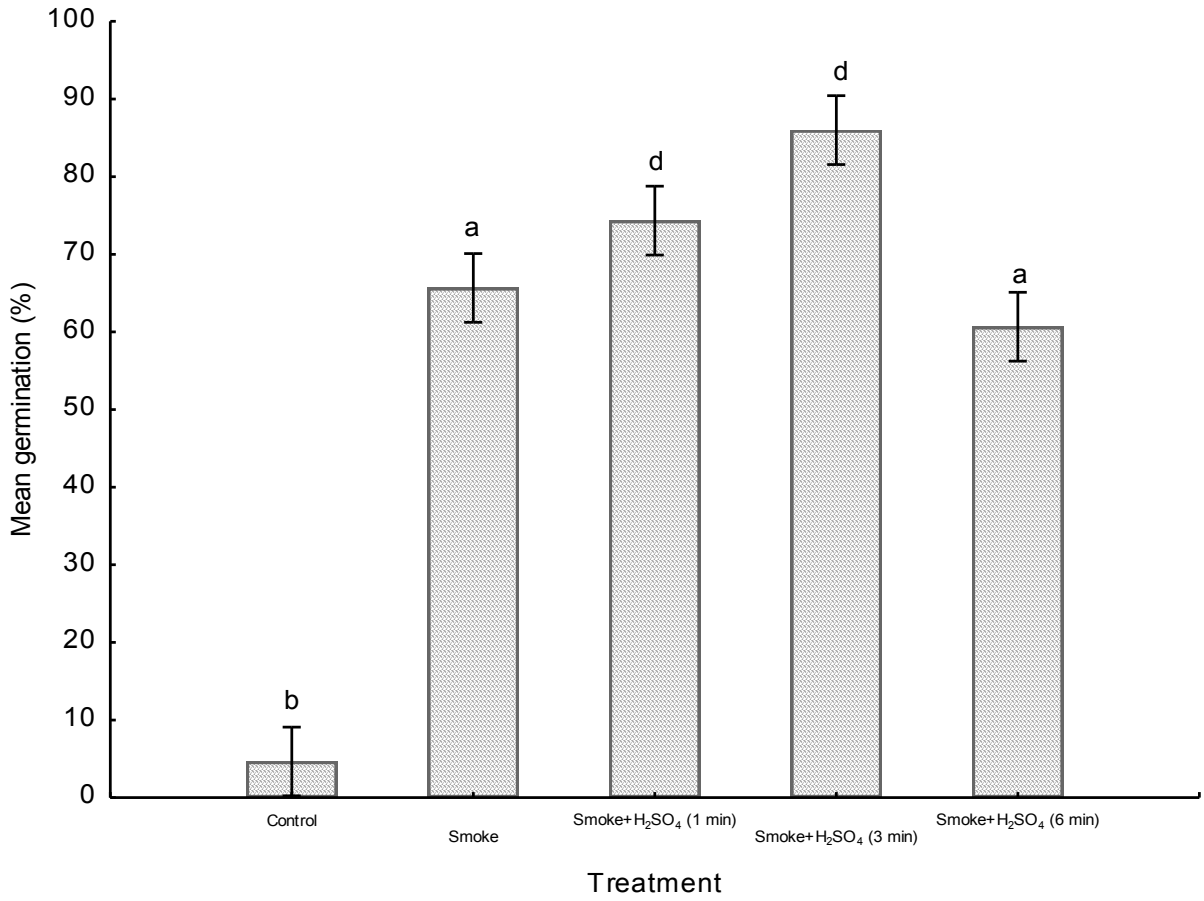

Figure 3.2 Effect of different treatments on the germination capacity of *Salvia runcinata.* Grey bars indicate the total number of seeds that germinated after 31 days. Different letters are included to show significant mean differences at the 95% confidence interval after Fisher LSD post-hoc analysis.

Table 3.1 A factorial ANOVA summary of the statistical result correlating interactions related to the control and various other treatments used to induce germination in *S. runcinata* seeds.

Treatment Interaction	***P*-value**
Control*Smoke	0.000013
Control*Smoke + H_2SO_4 (1 min)	0.000004
Control*Smoke + H_2SO_4 (3 min)	0.000000
Control*Smoke + H_2SO_4 (6 min)	0.003741

It is evident that smoke and scarification were useful in enhancing germination and worked synergistically. Not only does H_2SO_4 remove the testa of the seeds, but also somewhat the removal of the testa allows for imbibition and hydration of the seed, and, induces enzyme action to kick start germination (Gutiérrez-Nicolas and Zárete, 2008). According to Jain and co-workers (2008), scarification results in the breaking of the seed coat. Karrikin, which was isolated from plant-derived smoke water and identified by Van Staden and his colleagues (2004) as a highly active germination promoter, interacts in some way with endogenous PGRs controlling the physiological effects that lead to the germination of the seed which preceed plant development. The exact mode of action of the compound is however still unknown, but it is speculated to modulate (1) the sensitivity of the tissue to PGRs, (2) activate enzymes and/or (3) modifies the receptor molecules (Daws *et al.,* 2007; Light *et al.,* 2010).

3.4.2. Influence of explants and plant growth regulators

Seedlings provided a highly regenerative source of plant material for inducing cultures of *S. runcinata* with the first signs of shoot induction being evident after three weeks. Regardless of the amount and/or ratio of auxins and cytokinins used, axillary buds of nodal explants were highly organogenic with a significantly higher shoot formation capacity as compared to others (**Figure 3.4**). These explants also seemed to produce shoots more than other explants which generated a higher biomass (**Figure 3.5**). Nodal explants were also beneficial in generating cultures of

two Spanish endemic species of *Salvia* (Cuenca and Amo-Marco, 1999). Using 4.43 µM BA resulted in higher proliferation of shoots but the addition of growth regulators in this plant proved not to be imperative in promoting shooting. Here factorial ANOVA revealed that there was no interaction between the explant-type and PGRs used regarding the shoot number (**Table 3.2A**) and shoot weight (**Table 3.2B**).

Culture establishment appears to be species-dependent for *Salvia* species. Contrary to the data of Makunga and Van Staden (2008) for *Salvia africana-lutea* L., where a combination of 2.9 µM IAA with 4.4 µM or 8.9 µM BA was crucial for *in vitro* establishment, there was no need to supplement the growth medium with different PGRs as PGR-free medium could support production of shoots. This indicated that levels of endogenous hormones were sufficient in promoting organogenesis. Still, this is different from the *in vitro* organogenic potential of *S. fructicosa* Mill reported by Arikat *et al.* (2004). Those authors suggested that the addition of cytokinins (0.75 µM BA) played a vital role in bud break of nodal explants.

Regeneration from leaf explants was problematic as these explants proved not to be the best choice, producing fewer shoots. Also, an elevated incidence of browning which led to microplant death was higher (**Figure 3.4**). Similarly, stems had lowered capacity for adventitious shoot formation. *In vitro* shooting of *S. runcinata* seems to be dependent more precisely on the explant type than the PGR treatments. Sharma and Natuiyal (2009) emphasise the importance of choosing the best explant for culture initiation as plants often exhibit inter-explant variability for shoot promotion. Such variable responses for different explants type can be attributed to the physiological condition of the starter culture material, which is also determined by genetic factors (Nagarathna *et al.,* 1991; Dhar and Joshi, 2005).

All plants produced (from the current protocol) were healthy with no apparent signs of hyperhydricity as noted for *S. stenophylla* by Musaruwa *et al.* (2010), a plant belonging to the same species complex. Furthermore, ½ MS was used for all the experiments in this study and did not have a detrimental effect on the growth and

development of the plants. This is however contrary to results obtained by Shimomura and Kitazawa (1991) where low-ion strength MS medium caused poor growth and hypehydric shoots of *S. miltiorrhiza*. A higher occurrence of hyperhydration was reported by Hasoki and Tahara (1993) for *in vitro* multiplication of *S. leucantha* (a native Mexican perennial plant) with 1 mg l^{-1} (4.43 µM) BA. As there is no congruency regarding the micro-environmental conditions which are best for propagating sages, protocol development becomes an important criterion for micropropagation. This further supports the need to examine individual species as protocols produced for related species may not easily be adopted even though plants may be close relatives.

Microplants derived from the leaf and stem explants had fewer roots (0.281 and 0.875; respectively). Otherwise, rooting proved to be best for plantlets regenerated from nodal explants with generally 2.197 roots being associated with each nodal explant (P=0.00012) (**Figure 3.6**). All plants rooted easily on PGR-free medium obviating the need to investigate a separate rooting regime as most plants rooted within 23 days. This is highly beneficial for a commercial setup as it reduces labour time and costs associated with plants being transferred from one medium to the next to induce rooting.

Figure 3.3 *In vitro* propagation cultures of *Salvia runcinata* L.f.: (A) Germination of seeds on smoke and 3 min H_2SO_4 after a period of 31 days. Hypocotyl (h); cotyledon (c); primary leaf (pl), (B) Healthy shoots from nodal explants growing in PGR-free media, (C) Rooting from nodal explants in PGR-free medium, (D) Stem growth on a plant in PGR-free medium, (E) Root length on a plant in PGR-free medium, (F) Callus developing on wounded site of a shoot in PGR-free medium, (G) A week old plantlet transferred to PGR-free medium after 31 days of germination for further development, (H) Rooting of a 2 week old plantlet transferred from a smoke and 3 min H_2SO_4 medium to PGR-free medium, (I) Four week old plants acclimatised in a glasshouse, (J) Two month old plants acclimatised in a glasshouse which flowered in November 2011, (K) Acclimated plants had typical growth and developmental

characters, showing flowers which are similar to those plants that were not *in vitro* propagated.

Table 3.2 A factorial ANOVA summary of the statistical result correlating the effect of a combination of PGRs and the interaction they have with explant-type on shoot number and shoot weight.

	Effect	Degrees of freedom	*F* test	*P*-value
A. Shoot number	PGR combination	3	9.974	0.00001
	PGR combination*Explant	10	3.918	0.00010
B. Shoot weight	PGR combination	1	1.250	0.26557
	PGR combination*Explant	8	2.082	0.04180

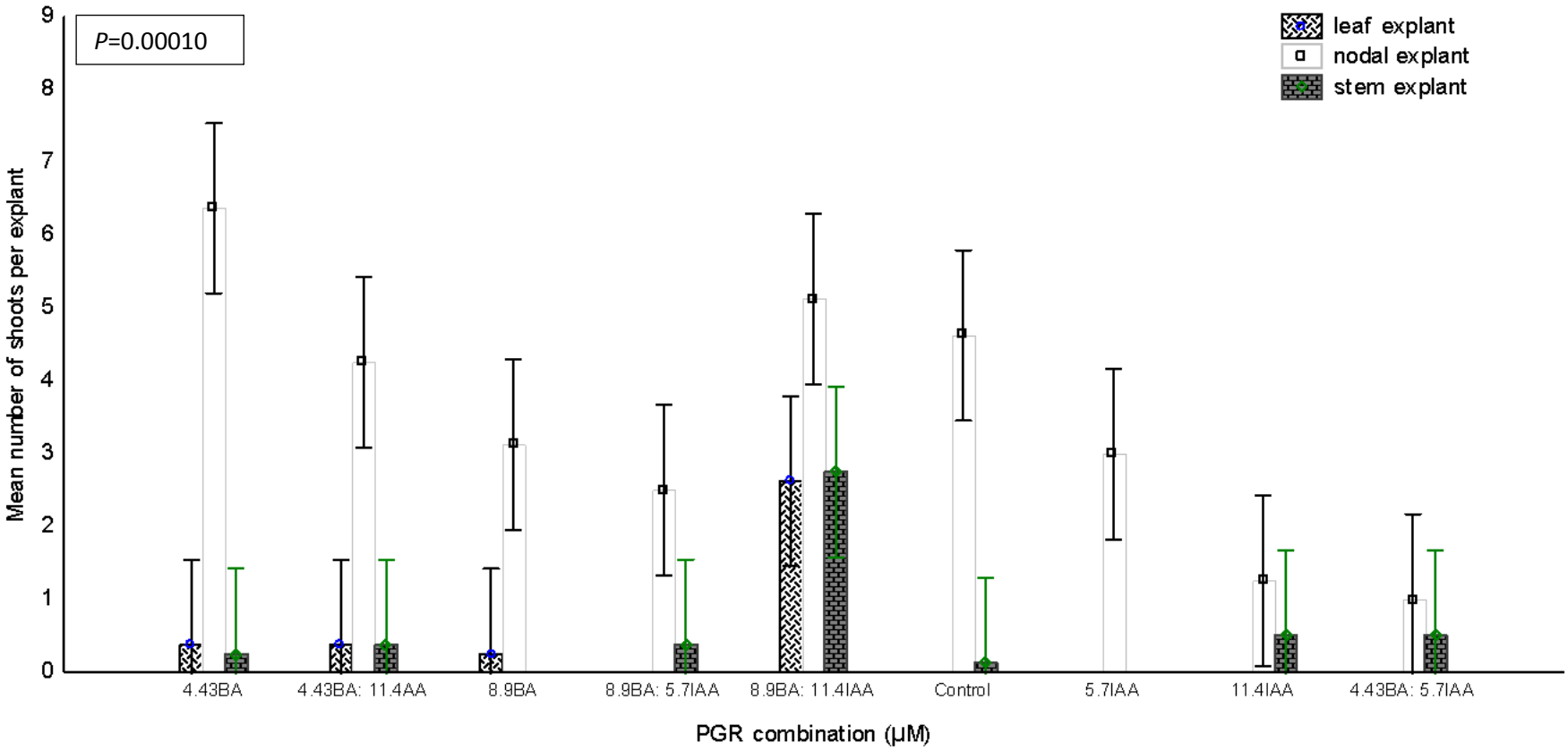

Figure 3.4 Influence of IAA and BA combinations on the production of shoots from leaf, nodal and stem explants of *Salvia runcinata*. Values represent the mean number of shoots per explants and vertical bars represent standard error at the 95% confidence interval.

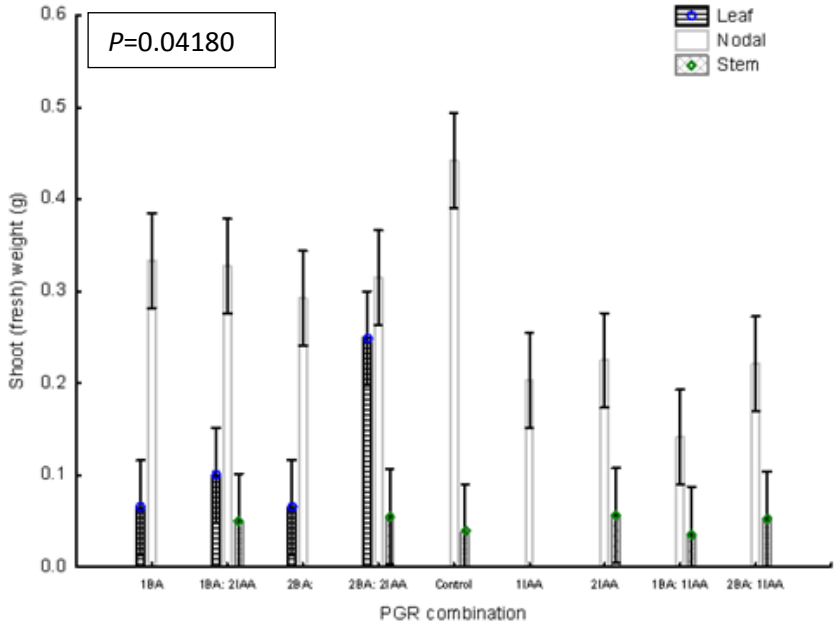

Figure 3.5 Influence of IAA and BA combination on shoot weight. Values represent the mean shoot fresh weight per explant and vertical bars represent standard error at the 95 % confidence interval.

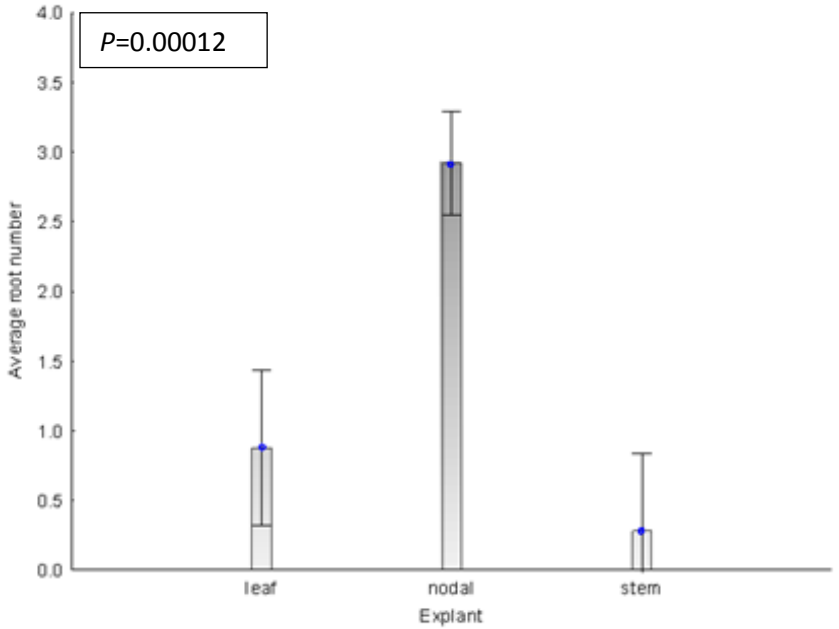

Figure 3.6 Effect of different explant-type on rooting frequency. Values represent the average root number per explant and vertical bars represent standard error at the 95 % confidence interval

3.4.3. Callus formation

Callusing was only observed on the cut ends of microshoot placed on hormone-free media (**Figure 3.3F**). The same phenomenon was observed on *Salvia africana-lutea* by Makunga and Van Staden (2008).

3.4.4. Acclimatisation

The acclimatisation protocol used for the *in vitro* rooted microshoots was successful. Out of the 20 potted plants housed in the glasshouse, 80% survived. Normal growth of the plants was observed throughout the growth phases in the glasshouse. There were no obvious morphological abnormalities or variations noted up to and including during flowering stages of the plants. High acclimation are normally typical for micropropagated plants of *Salvia species* with survival rates greater than 70% (Cuenca and Amo-Marco, 2000; Arikat *et al.,* 2004; Avato *et al.,* 2005; Miśić *et al.*, 2006; Matu *et al.,* 2006; Makunga and Van Staden, 2008; Musarurwa *et al*., 2010 and others).

3.5. Conclusion

The data presented here demonstrate the establishment of an efficient *in vitro* germination and micropropagation protocols for *Salvia runcinata* allowing the regeneration of new plants. The use of nodal explants is beneficial than other explant-types and is therefore recommended as 6 viable plants can be obtained per nodal explant within a four month period. This therefore can be a feasible protocol for large scale production for commercialisation of *S. runcinata* as it would yield 1296 to 46656 viable plants in 4 to 6 months from one nodal explant. This should support the pharmaceutical and cosmeceutical industry in the event of *S. runcinata* being used as an alternative source for epi-α-bisabolol. This thus makes this protocol suitable for *ex situ* conservation of *S. runcinata* plantlets.

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Chapter 4

Agrobacterium-mediated transformation of *Salvia runcinata* L.f.

4.1. Introduction

Today, plants are still considered as the major source of high-value products used in the speciality chemicals industry (pharmaceuticals, agrochemicals, flavours, fragrances, dyes, oils, resins and biopesticides) (Kim *et al.,* 2002). All this can be ascribed to a large and diverse group of chemicals including alkaloids, anthraquinones, anthocyanins, flavonoids, saponins and terpenes produced by plants through secondary metabolism (Srivastava and Srivastava, 2007). Secondary metabolites are compounds that are biosynthetically derived from primary metabolites but more limited in distribution to the plant kingdom, being restricted to a particular taxonomic group (Balandrin *et al.,* 1985). In contrast to primary metabolites, secondary metabolites are not essential for plant growth, are frequently accumulated by plants in small amounts (less than 1% dry weight), and tend to be synthesised in specialised cell types and at distinct developmental stages, e.g. trichomes, ducts, canals, laticifers (Kim *et al.,* 2002) or due to specific environmental cues.

Currently, most pharmaceutically important secondary metabolites are isolated from wild or cultivated plants because their chemical synthesis is not economically viable (Oksman-Caldentey *et al.,* 2004). For example, taxol, a complex diterpene alkaloid found in the bark of the Taxus tree has been a highly successful anticancer drug due to its unique mode of action on the microtubular cell systems. This substance interferes with cell division, thus the rapidly dividing cancer cells become severely hampered as they grow (Nicolaou *et al.,* 1994; Expositó *et al.,* 2009). The major challenge in the production of this compound in large amounts was attributed to the

scarcity of the Taxus tree, the enormous commercial value and the economics of the synthetic process of taxol (Vanisree *et al.,* 2004). Nowadays, taxol is produced only through use of plant cell cultures, with other eukaryotic systems, such as yeast being explored for future use. The biotechnological production of commercially important compounds (including taxol) in cell and organ cultures has been explored and considered to be an alternative to agricultural processes. Cell cultures, however, are genetically unstable and cultured single plant cells tend to produce low yields of secondary metabolites, some compounds are not synthesised if the cells remain undifferentiated (Giri and Narasu, 2000). Most efforts now focus on the production of transformed roots using a natural vector system such as *A. rhizogenes* to induce 'hairy root syndrome' (Cônsolli *et al*., 1995; Christey, 2001).

Hairy roots have several properties that promoted their use for various plant biotechnological applications. These transformed root cultures are differentiated and are phenotypically and genetically stable, display a rapid growth rate independent of any source of exogenous hormone and are characterised by their lack of geotropism (Giri and Narasu, 2000; Christey and Braun, 2004; Georgiev *et al.,* 2007). Hairy roots of some plants are known to produce novel secondary metabolites that are not normally present in plants, and therefore, represent a possibility for the discovery of new biologically important compounds (Dhakulkar *et al.,* 2005). Most importantly, to emphasize, these root cultures are easy to maintain and exhibit about the same or a greater biosynthetic capacity for secondary metabolite production compared to their mother plants (Kim *et al.,* 2002), proving to be more economical for commercial production purposes.

Attempts have been successfully made to establish hairy root cultures of plant species belonging to the family Lamiaceae using *A. rhizogenes* including *Salvia miltiorrhiza*, *S. officinalis, S. sclarea* and other *Salvia* species. This is due to their richness in terpenoids and other related secondary metabolites (**Table 4.1**). The hairy roots of *Salvia miltiorrhiza* (among the species mentioned above) are mostly exploited in China due to their high-yield during tanshinone production that is used to treat several diseases (Hu and Alfermann, 1993). The strategy of hairy root

production is now moving from laboratories to large-scale industrial production through the use of bioreactor systems (Guillon *et al.,* 2006).

In this chapter, we report on the transformation and induction of hairy roots in *Salvia runcinata* using *A. rhizogenes* agropine strains. To the best of our knowledge, this part of the study describes for the first time, the establishment of *Salvia runcinata* hairy root cultures obtained after transformation with the *A. rhizogenes* strains A4T and LBA 9402. This then is followed with the characterisation of the chemical profiles of hairy roots for secondary metabolites (described in Chapter 5).

Table 4.1 Progress on research using *Agrobacterium rhizogenes*-mediated transformation on *Salvia* species for the production of secondary metabolites.

***Salvia* species**	**Plasmid strain**	**Investigated product**	**Plant regeneration**	**Reference**
Salvia africana-lutea	LBA 9402	-	No	Ramogola *et al.,* 2008
Salvia broussonetti	ATCC 15834	Diterpenes	No	Fraga *et al.,* 2005
Salvia miltiorrhiza	ATCC 15834	-	Yes	Zhang *et al.,* 1997
	LBA 9402	-		
	LBA 9402	Diterpenoids	No	Hu and Alfermann, 1993
	ATCC 15834	-		
	TR 105	-		
	R 1601	-		
	A 4 1027	-		
	ATCC 15834	Lithospermic acid B, rosmarinic acid and other related phenolic compounds	No	Chen *et al.,* 1999; Chen *et al.,* 2001; Yan *et al.,* 2006
	ATCC 15834	Tanshinones	No	Wang *et al*., 2007
Salvia officinalis	ATCC 15834	Rosmarinic acid	No	Grzegorczyk *et al.,* 2006
	A4	-		
Salvia sclarea	LBA 9402	Diterpenoids and triterpenoids	No	Kuźma *et al.,* 2006; Walencka *et al.,* 2007
Salvia tomentosa	ATCC 15834	Phenolic compounds and flavonoids	No	Marchev *et al.,* 2011

[1]This table is not necessarily exhaustive and data were extracted from Scopus (www.scopus.com) and Google Scholar databases (www.scholar.google.com).

4.2. Materials and Methods

4.2.1. Bacterial strains

Colonies of *Agrobacterium rhizogenes* agropine strains: A4T and LBA 9402 were routinely grown on yeast extract peptone (YEP) agar medium (10 g l^{-1} peptone; 10 g l^{-1} yeast extract; 5 g l^{-1} NaCl solidified with 15 g l^{-1} agar, pH 7.0, pH adjusted to 5.8 with 1 M NaOH or 1 M HCl) supplemented with 250 µM acetosyringone (3', 5'-dimethoxy-4'-hydroxyacetophenone, Sigma, Germany) and rifampicin (Rimactane®, Sandoz Ltd, South Africa) (100 mg l^{-1}) in the dark, at 28 °C for 24 h using standard microbial streaking techniques.

Liquid cultures were initiated by inoculating a single colony of each of the two strains in 50 ml flasks with YEP medium supplemented with rifampicin (100 mg l^{-1}) and 250 µM acetosyringone. Liquid cultures were shaken in the dark at 28 °C at 130 revolutions per minute (rpm) for two days or until the bacteria had grown to the saturated phase (OD_{600} = 1-2). Prior to transformation, 13 ml of the saturated bacterial solution was transferred to a 15 ml centrifuge tube (Sterilin, UK) and centrifuged in a desktop centrifuge (Sigma 302) at 4 000 rates per minute (rpm) at 20 °C for 15 min. The supernatant was discarded; the pellet washed and resuspended in 15 ml liquid Murashige and Skoog (MS) medium (0.1 g l-1 myo-inositol; 3% [w/v] sucrose) to remove the antibiotics. The MS salts were prepared by Highveld Biologicals and for each litre, the manufacturer's recommend using 4.4 g of the powder for full complement of macro- and micronutrients including vitamins (**Appendix A**[1]). The bacterial solution was centrifuged once more and the pellet was resuspended in 90 ml liquid MS medium.

4.2.2. Transformation and establishment of hairy root cultures

In vitro derived nodal (1 cm) and leaf (1 x 1 cm) explants from two month old *Salvia runcinata* seedlings/plantlets were incubated for 10 min with freshly grown *A. rhizogenes* cultures. Thereafter, the explants were blotted dry and transferred to PGR-free ½ MS medium containing 250 µM acetosyringone and co-cultivated for two

days in darkness. After two days, the explants were washed twice for 20 min per wash in sterile distilled water containing 250 µg ml^{-1} cefotaxime (Claforan from Aventis, South Africa). The explants were then blotted dry on sterile tissue paper and transferred onto Petri dishes (100 cm X 2 cm from BD Falcon™) (4 explants per plate) containing solid ½ MS medium with 250 µg ml^{-1} cefotaxime. The plates were placed in the dark at 24 °C ± 2 °C growth room and monitored daily for root development. When roots appeared and grew to a length of 1 cm, they were cut and transferred with a fragment of tissue to fresh PGR-free ½ MS medium with cefotaxime; each individual regenerating root from an explant was regarded as arising from a single transformation event. Axenic cultures were established after 4 to 6 subcultures and each subculture was done after every 2 weeks. Bacteria-free putative hairy roots were then maintained on both solid and liquid PGR-free ½ MS medium, in the dark at 24 °C ± 2 °C on a rotary shaker (150 rpm) for liquid cultures. In all subsequent experiments, liquid cultures were obtained by incubating four 2 cm long root tips in 250 ml Erlenmeyer flask containing 50 ml ½ MS medium. Subcultures were made every three weeks. Fresh weight of the root cultures in liquid medium was recorded before initiation of suspension cultures (0.05 g) and after a period of thirty days after culture initiation to measure biomass accumulation.

4.2.3. Detection of bacterial DNA in putative transgenic root tissue

4.2.3.1. Extraction of genomic DNA (CTAB method)

Plant genomic DNA for the polymerase chain reaction (PCR) analysis was extracted using the standard cetyltrimethylammonium bromide (CTAB) method for RNA by White *et al.* (2008) modified to include the step of DNA precipitation. Fresh putative root tissue (0.1 g) of A4T3, A4T5 and wild type were ground to a fine powder in liquid nitrogen using a pestle and a mortar. The ground tissues were transferred into three separate 2 ml microcentrifuge tubes with 1.2 ml preheated (65 °C) extraction buffer (2 % (w/v) CTAB, 2 % (w/v) polyvinyl pyrrolidone (PVP), 100 mM Tris-HCl (pH 8.0), 25 M NaCl, 0.5 g L^{-1} spermidine containing 3% (v/v) β-mercaptoethanol (added immediately prior to extraction to preheated autoclaved buffer) then vortexed thoroughly for 1 min. The tubes were incubated in 65 °C water bath for at least 30 min with constant vortexing. The samples were centrifuged at 5 000 rpm for 10 min

in an ultrucentrifuge (Sorval RC26 Plus) and the supernatant was transferred to new 2 ml microcentrifuge tubes. An equal volume of chloroform: isoamyl-alcohol (24:1; v/v) solution was added to the supernatant followed by a vortex step for 1 min at 4 °C and centrifuging step at 5000 rpm for 15 min. The aqueous phase was then transferred to new 2 ml microcentrifuge tubes and the chloroform: isoamyl-alcohol extraction was repeated. Precipitation of the DNA was done by adding (1/10 th of the volume of the supernatant) 4.4 M ammonium acetate (105 ml H_2O, 50.5 ml glacial acetic acid, 45 ml NH_4OH; pH 5.2) and an equal volume of ice-cold isopropanol. The tubes were briefly inverted to precipitate the DNA and stored at -20 °C overnight. The DNA was collected by centrifuging at 5 000 rpm for 30 min followed by the removal of the supernatant and washing of each pellet with 1 ml 70 % (v/v) EtOH then 1.5 ml 100 % (v/v) EtOH. The DNA was then left to air-dry for at least 20 min, and subsequently resuspended in 50 µl de-ionised distilled water (ddH_2O).

This method however yielded DNA of low quality with high amounts of polyphenols and polysaccharides which normally interfere with enzymatic reactions such as PCR and endonuclease restriction digestion for Southern blot analysis. Extraction using an Invisorb® Spin Plant Mini kit (described below) was therefore later adopted to improve the quality of DNA.

4.2.3.2. Extraction of genomic DNA (using an Invisorb® Spin Plant Mini Kit)

The quality and the amount genomic DNA is important for successful Southern blotting. High quantities of polyphenols and polysaccharides binding to the genomic DNA promote its degradation and render it unrestrictable and un-amplifiable in PCR reactions (Hanania *et al.,* 2004). To deal with this problem, an Invisorb® Spin Plant Mini Kit was utilised to generate purer samples. Briefly, fresh putative roots (60 mg) of A4T3, A4T5 and wild type, independently, were ground using liquid nitrogen to a fine powder. The ground powder was transferred into three separate 1.5 ml reaction tubes with 400 µl lysis buffer P and 20 µl proteinase K (provided in the kit), then vortexed briefly. The tubes were incubated at 65 °C for 30 min. The lysis solution was transferred onto the prefilter membrane and this was then placed into a 2 ml

receiver tube and both units were centrifuged for 1 min at 12 000 rpm. The prefilter membrane was discarded and to remove RNA, 40 µl of RNase A (10 mg ml^{-1}) was added to the filtrate followed by brief vortexing prior to incubation at room temperature for 5 min. Subsequently 400 µl of the binding buffer P was added and the samples were vortexed thoroughly. To bind the DNA, the suspensions were transferred onto spin filters placed into 2 ml receiver tubes and incubated for 1 min. The DNA was collected by centrifuging (12 000 rpm) for 1 min, the filtrate was discarded. The spin filters were then placed into 2 ml receiver tubes and washed twice with 550 µl of wash buffer I. After each wash, the extracts were centrifuged at 12 000 rpm for 1 min. Finally, the filtrate was discarded and to remove the residual alcohol, the spin filters were air-dried for at least 20 min. To elute the DNA, 100 µl of pre-warmed (65 °C) elution buffer D was added. The DNA was collected by centrifuging at 10 000 rpm for 2 min.

4.2.3.3. Extraction of RNA for cDNA synthesis

Total RNA was extracted using the standard CTAB method by White *et al.* (2008) as outlined in Section 4.2.3.1 excluding the DNA precipitation step. To confirm the transcription of the inserted *rol* and *ags* genes from *A. rhizogenes* T-DNA, the cDNA was synthesised using RNA from both the tissue culture plant roots (negative control) and the transgenic hairy roots (Revertaid™ H minus First strand cDNA synthesis kit was used). The protocol from the kit was followed according to the manufacturer's instructions. Briefly, 500 ng of total RNA from A4T3, A4T5 and wild type clones were independently mixed with 1 µl oligo $(dT)_{18}$ in 1.5 ml sterile nuclease-free microcentrifuge tubes and nuclease-free water was added to bring to a total volume of 12 µl per sample. The samples were centrifuged for 1 min at 12 000 rpm prior to incubation at 65 °C for 5 min. Subsequently samples were chilled on ice for 2 min followed by spinning down for 1 min then put back on ice. The reverse-transcription mix consisting of 4 µl 5X reaction buffer, 1 µl RiboLock™ RNase inhibitor, 2 µl dNTP mix and 1µl RevertAid™ H Minus M-MuLV Reverse Transcriptase was prepared and added to each sample on ice bringing the volume to 20 µl. The samples in the tubes were spun down for 1 min then incubated at 45 °C for 1 h. The reaction was then terminated by increasing the temperature to 70 °C and

samples were further incubated for 10 min. The tubes were left at -20 °C prior to use for RT-PCR.

4.2.3.4. PCR amplification of *rol* and *ags* genes in the root DNA

The plasmid DNA which was isolated from *Agrobacterium rhizogenes* strain (A4T) by the alkaline lysis method as outlined by Li *et al.* (1995) was donated by Miss J. Colling and used as a positive control. Genomic DNA from tissue culture plant roots (negative control) and DNA from putative transgenic clones were all subjected to PCR *a*mplification. A PCR reaction without the DNA was also used as a negative control. In order to confirm transgenesis, oligonucleotide specific primers for PCR detection of homologous sequences to *rol* A, *rol* B and *rol* C (on the T_L-DNA region) and *ags* gene (on the T_R-DNA region) from *A. rhizogenes* T-DNA were used (all from Integrated DNA technology (IDT), USA) (see **Table 4.2** for sequences). For each 20 µl reaction, the optimal concentrations of reagents listed in **Table 4.3** were used for amplification. The PCR cycling conditions consisted of an initial denaturation of the template at 94 °C for 3 min for the first cycle followed by a denaturation step at 94 °C for 1 min (35 cycles), with primer specific annealing temperatures (**Table 4.2**); and an elongation step at 72 °C for 2 min 30 sec with a final 7 min extension step at 72 °C. Amplifications were conducted in a 96 well GeneAmp® PCR system 9700 (Applied Biosystems, USA). The amplified products (20 µl) were analysed and viewed after electrophoresis on a 1% (w/v) agarose- tris-borate-ethylenediaminetetraacetic acid (TBE) gel with a *Pst* DNA ladder (generated by digesting 1 µg Lambda DNA with *Pst* I). Bands were visualised by using 1 µg ml^{-1} ethidium bromide (EtBr) staining.

Table 4.2 Primer sequence, size and annealing temperatures used for the PCR amplification to detect the *rol* and *ags* genes incorporated in *S. runcinata* hairy root DNA.

Primer	Sequence	° C	Size (kb)
rol A	5'- CAG AAT GGA ATT AGC CGG ACT A -3' and 5'- CGT ATT AAT CCC GTA GGT TTG TTT -3'	54	0.3
rol B	5'- ATG GAT CCC AAA TTG CTA TTC CTT CCA GA-3' and 5'- TTA GGC TTC TTT CTT CAG GTT TAC TGC AGC -3'	54	0.4
rol C	5'- CAT TAG CCG ATT GCA AAC TTG -3' and 5'- ATG GCT GAA GAC GAC CTG -3'	54	0.6
ags	5'- CGG AAA TTG TGG CTG TTG TGG AC -3' and 5'- AAT CGT TCA GAG AGC GTC CGA AGT -3'	59	1.6

All primers were purchased from IDT (USA)

Table 4.3 PCR reaction for the amplification of the *rol* and *ags* genes.

Reagent	Concentration	Company
Taq Buffer (NH_2SO_4)	1X	Fermentas
Taq Polymerase	2.5 u	Fermentas
dNTPs	0.2 mM	Fermentas
$*^{32}P$	10 µCi	Promega
$MgCl_2$	1.5 mM	Fermentas
Primer (forward and reverse)	0.2 mM	IDT
Template	25 ng	-
Ultra pure H_2O (to make total volume of 20 µl)		-

* This was only added in the PCR cycle for the probe preparation and labelling

4.2.3.5. Probe preparation and labelling

Plasmid DNA (A4T) donated by Miss J. Colling was used as a PCR template to amplify *rol* A gene using specific primers as outlined in **Table 4.2.** The PCR labelling was conducted using 1 mM [α-^{32}P] dATP 10 µCi/mmol 0.025 µl of 50 mM Tricine (pH

7.6) (Promega, USA) using the same PCR cycle and reaction as outlined in Section 4.2.3.4. and Table 3 respectively. The resultant amplicons were purified using the PCR purification Kit (Qiagen, Germany) according to the manufacturer's instructions.

4.2.3.6. Southern hybridisation

DNA was extracted as described in Section 4.2.3.2 and 20 µg of the genomic DNA was digested at 37 °C to completion (24 to 72 h) using suitable amounts of the restriction endonuclease *Eco*R1 (Fermentas) according to the supplier's recommendations. Plasmid DNA (25 ng) was also completely digested and used as a positive control. The DNA which was extracted and digested from non-transgenic tissue cultured plant roots was used as a negative control. All the restriction digests were electrophoresed on 0.8% (w/v) agarose gel at 60 V for 4 h. To transfer the DNA from the gel to the Hybond^{TM} –N+ membrane (Amersham, UK), a rapid downward method by Koetsier *et al.* (1993) was used. The blotting membrane and one layer of filter paper were cut to the size of the gel and the top left corner of the membrane was cut to indicate the first lane. Another filter paper was cut to be 1 to 2 cm larger than the size of the gel. The membrane and the filter papers were placed in 10 X SSC (15 M NaCl, 1.5 M sodium citrate, pH 7) prior to transfer. The depurinated gel was placed on a base of a Petri dish with the DNA side facing upwards. The blotting membrane was carefully placed on top of the gel and a clean glass rod was used to roll out all the bubbles. The filter paper which was cut to be 1 to 2 cm larger than the gel was carefully placed on top of the membrane and smoothed using the clean glass rod. An 80 mm stack of paper towel (1 to 2 cm larger than the gel) was placed on top of the gel-membrane stack and the entire pile was inverted and the gel was slid off the Petri dish. The filter paper which was the same size as the gel was placed on top of the gel. A wet wick (a double layer of wet filter paper) was folded to the same size as the gel and placed on top of the gel to avoid it from drying. The transfer was done overnight. To confirm the success of the transfer, the gel was viewed under UV light. The membrane was crosslinked under UV light to ensure that the transferred nucleic acids adhered to the membrane using ultraviolet cross-linker at 120 mJ cm^{-1} for 2.5 min (Ultra.Lum, Scientific Associate, USA).

4.2.3.7. Pre-hybridisation and hybridisation

The membrane was pre-hybridised at 42 °C in Rapid Hyb buffer (GE Health-care, UK) using a hybridisation oven (Amersham, UK) with continuous constant rotation. The pure radioactively labelled PCR product was meanwhile denatured by heating at 100 °C for 10 min in a 96-Well GeneAmp® PCR System 9700 (Applied Biosystems, USA). Subsequently, the 20 µl PCR product was snap-cooled on ice for one min to prevent the two DNA strands from annealing prior use. This volume of DNA was then added directly to the pre-hybridisation buffer in the hybridisation tube to initiate the hybridisation. The hybridisation of the probe to the membrane was conducted overnight at 54 °C with continuous constant rotation. After hybridisation, the membrane was washed twice for 5 min with 2 X SSC (3 M NaCl, 0.3 M sodium citrate, pH 7) containing 0.1% (w/v) SDS at room temperature. This was conducted twice for 5 min with 2 X SSC containing 0.1% (w/v) SDS for 20 min at 42 °C and the last wash lasted 15 min with 0.5 X SSC containing 0.1% (w/v) SDS at 65 °C.

4.2.3.8. Data collection and statistical analysis

Statistical analyses were performed using STATISTICA data analysis software system version 10 (StatSoft, Inc, Tulsa, USA). A random block design was used for all experiments and all experiments were repeated three times.

Four explants (leaf and nodal explants) were placed per Petri dish and there were twelve Petri dishes per treatment (strain). Each explant represented a replicate (N = 48). The explants were monitored daily for root development for a period of 31 days to determine the mean number of putative roots per explant for each strain used. The data for frequency of rooting were arcsine transformed prior to analysis using factorial ANOVA test. Differences between means reaching a minimal confidence level of 95 % were considered as being significantly different.

For biomass accumulation of hairy roots, four flasks of each clone were prepared and each flask represented a replicate. The fresh mass of the roots were determined

after 30 days. A t-test was used to analyse the results for fresh mass accumulation data.

4.3. Results and Discussion

4.3.1. Establishment of hairy root cultures

Infectivity of the agropine strains was determined by comparing their capability to induce putative rooting on target explants. As expected, pathological responses to infection by *A. rhizogenes* were noted after 3 to 4 weeks of inoculation and hairy roots exuded from the cut/wounded edges of the explants inoculated with A4T and LBA 9402 strains (**Figure 4.1**) (Tepfer, 1984; Giri *et al.*, 1997; Królicka *et al.,* 2001; Le Flem-Bonhomme *et al.,* 2004).

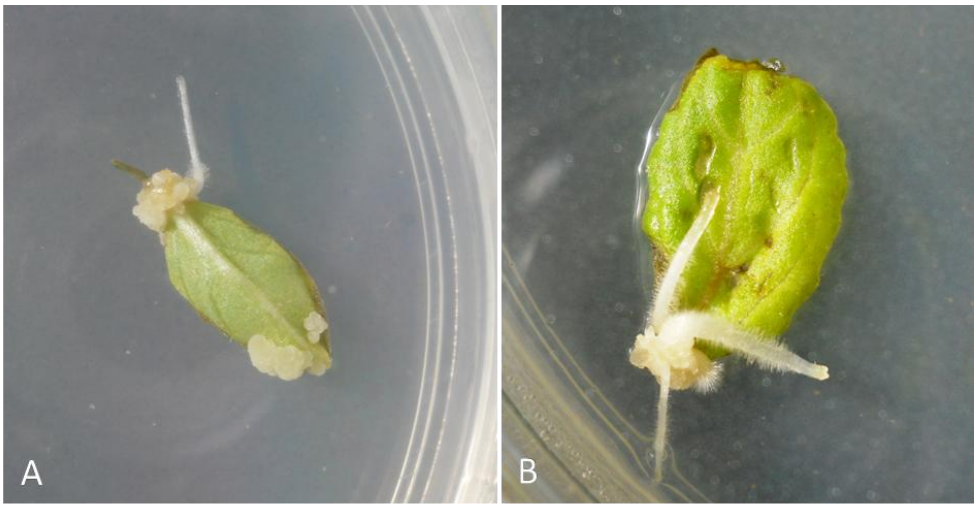

Figure 4.1 Induction of hairy roots on *S. runcinata* (A) Leaf explant inoculated with the A4T3 strain (B) Leaf explant inoculated with the LBA 9402 strain.

The frequency of hairy root induction was dependent on the strain with the LBA 9402 strain producing significantly more roots on each explants compared to the A4T strain (P=0.0075) (**Table 4.4**). Generally, infection by different strains is species

specific, for instance using LBA 9402, over ten hairy roots per explant were recorded by Akramian *et al.* (2008) on *Hyoscyamus reticulates* plants.

Table 4.4 Effect of using two different agropine strains (*Agrobacterium rhizogenes* LBA 9402 and A4T) on the induction of putative hairy roots using two different explants (leaf and nodal explant). Kruskal-Wallis ANOVA comparison of mean ranks (H = 11.97, df=3, N=20, *P*= 0.0075). The values denote multiple comparisons of mean ranks for all different strains on different explants types.

a

Strain	**Root number per explant**
A4T	
Leaf	2.2 ab
Nodal	0 a
LBA9402	
Leaf	10.4 b
Nodal	0.4 ab

b

		Leaf		**Nodal**	
		LBA	A4T	LBA	A4T
Leaf	LBA		0.129218	0.270125	0.025443*
	A4T			1.000000	1.000000
Nodal	LBA				1.000000
	A4T				

Different letters represent values that are statistically different. Asterisks indicate *P* values that showed statistical differences at 95% confidence limit

However, the LBA 9402 root putative transgenic lines irrespective of explant type used for the transformation grew slowly. In addition these tended to lose the hairy root phenotype, becoming extensively nodulated (**Figure 4.2**). They could not maintain their growth, turning brown and eventually died within the first four weeks of growth. Several reasons may explain the loss of the hairy root phenotype. Presumably, this loss could be as a result of deletion of genes that confer the hairy root characteristics within T-DNA. As pointed out by Chandra (2012) differential loss of T-DNA genes during transformation does occur. This loss drastically affects the phenotype and the growth patterns of the hairy roots, possibly explaining the phenomenon observed with the LBA 9402 lines. The effect of the culture conditions on the expression of the *rol* genes and the plant genotype are an important consideration. Also, the production of hairy roots may be linked to the expression of the T-DNA *rol* genes but these genes may not be stably integrated and eventually become displaced from the chromosomal regions where they were initially incorporated (Gelvin, 1990). Sometimes the T-DNA genes get into a chromosomal region that does not provide the correct chromosomal architecture for gene expression resulting to a negative transformation occurrence. T-DNA insertion into the plant genome is still poorly understood and the mechanisms involved are not well resolved.

In contrast to the LBA 9402 putative transformants, the A4T root lines derived from the leaf explants grew rapidly on PGR-free medium displaying a typical hairy root phenotype characterised by high degree of lateral branching and a lack of geotropism. Bandyopadhyay *et al.* (2007) also observed the same phenomenon as described here with A4T root lines displaying typical transformed growth while LBA root lines dedifferentiated and formed callus. In total, two putative root lines survived from the A4T strain and none survived from the LBA 9402 strain. The growth characteristic of the transformed roots was different from roots excised from tissue culture plants as non-transgenic roots died in the absence of PGRs. This striking difference in morphological phenotype and growth patterns is well established in many other species transformed using *A. rhizogenes* (Foo *et al.,* 2005). Only A4T lines readily established about three months after inoculation as axenic hairy root

cultures. Residual agrobacterial infection may be problematic but cultures were free of bacterial contamination.

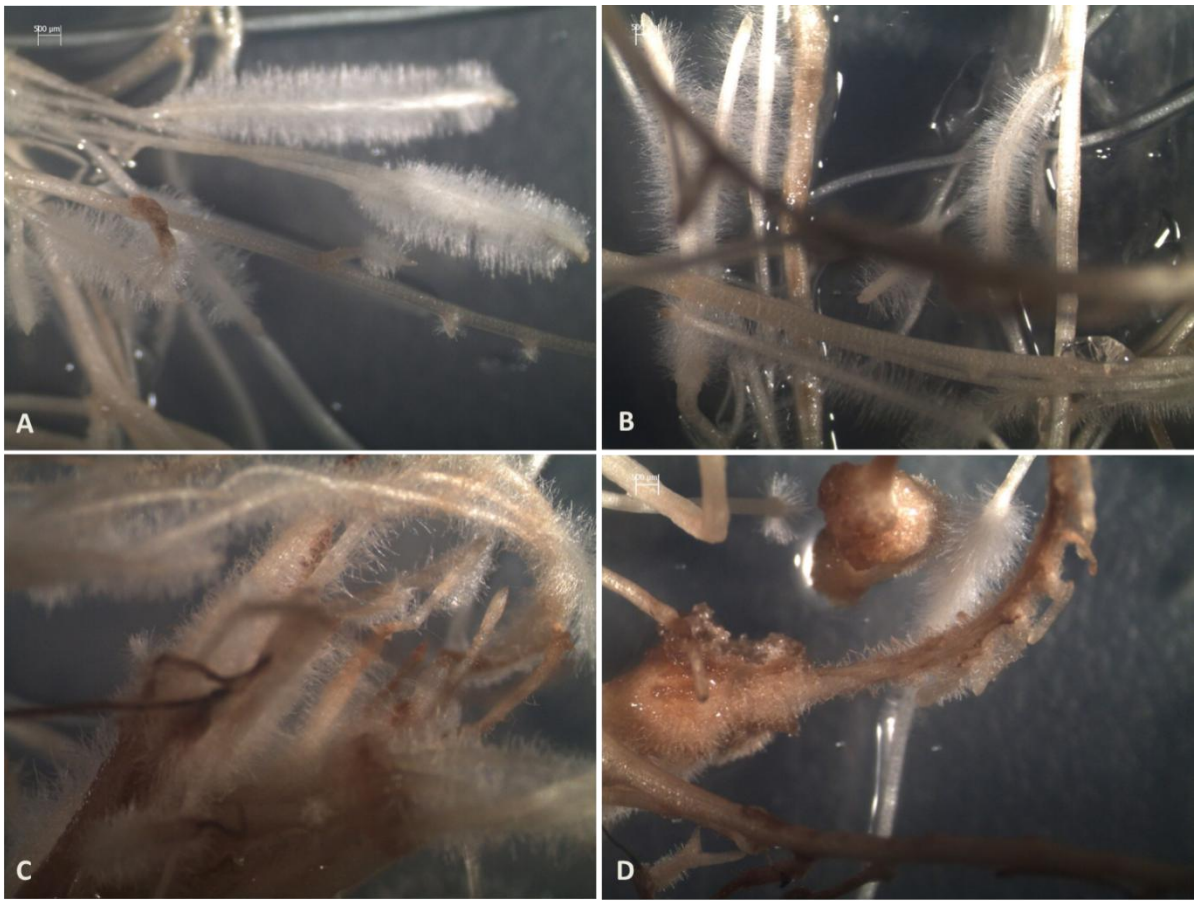

Figure 4.2 Characteristic phenotypes of *Salvia runcinata* hairy roots established using *Agrobacterium rhizogenes* A4T and LBA 9402 strains. (A) A4T3 clone; (B) A4T5 clone; (C) LBA 9402a clone; (D) LBA 9402a clone forming nodules.

4.3.2. Growth patterns of hairy root lines

The growth performance of the A4T5 clone on ½ MS PGR-free liquid medium was significantly higher than the growth of clone A4T3 (P=0.0238) as the A4T5 clone showed the highest biomass accumulation at the end of four weeks (2.66 g from the initial 0.05 g). Only 0.14 g biomass was accumulated by clone A4T3, representing a 2.8-fold increase in biomass (**Figure 4.3**).

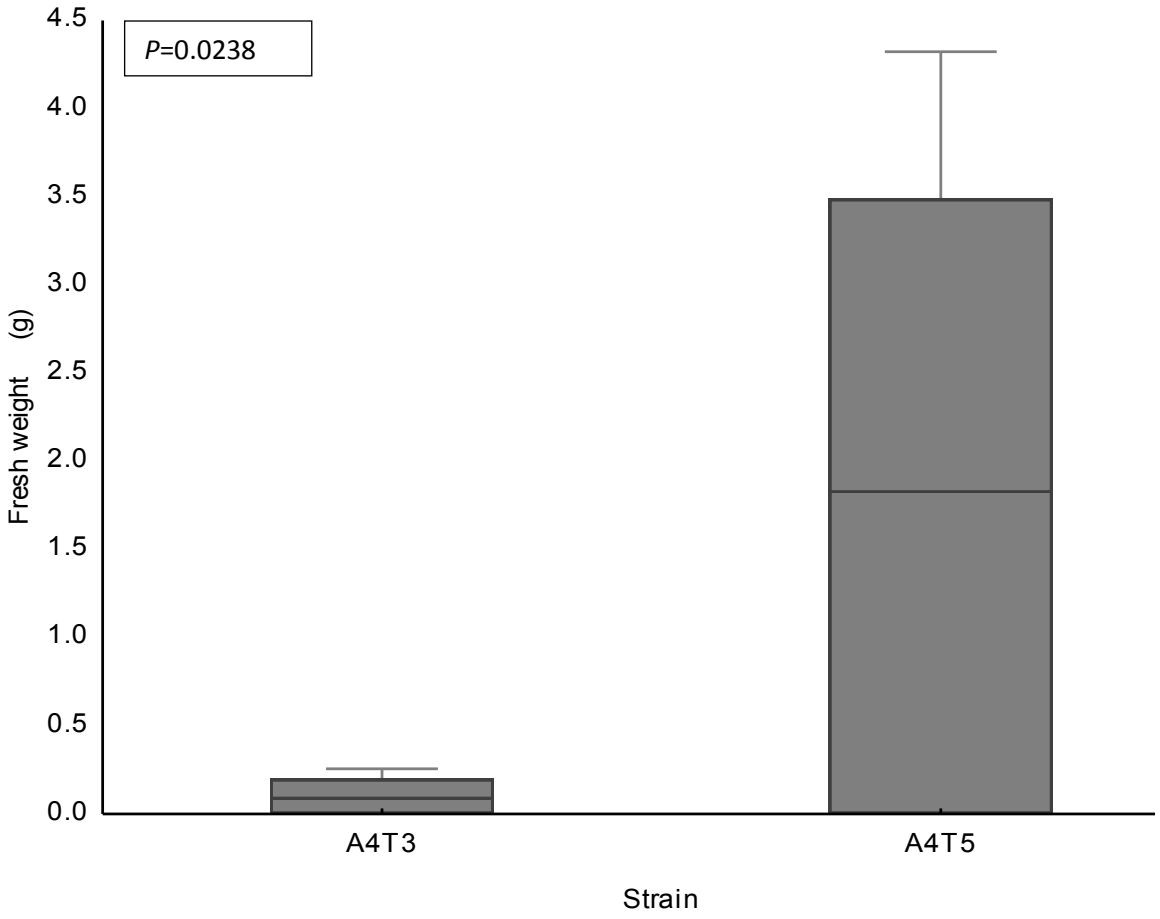

Figure 4.3 Growth of hairy root in suspension culture after four weeks. The initial inoculum for both lines was 0.05 g. Results represent mean of four replicates. Error bars represent standard errors calculated at the 95 % confidence interval.

4.3.3. Molecular analysis of clones

4.3.3.1. DNA isolation

The standard CTAB method primarily used for the isolation of the DNA yielded low quality DNA. This is mainly ascribed to the high amounts of polyphenols and polysaccharides in *Salvia* species (Loo and Foo, 2002; Abu-Romman, 2011). These are the same compounds that bind firmly to nucleic acids during the isolation and interfere with consequent reactions. The isolated DNA fast becomes a brown colour due to the polyphenols discharged from the vacuole during the cell lysis process and these polyphenols undergo rapid oxidation, irreversibly interacting with nucleic acids (Verma *et al.,* 2007). The Invisorb® Spin Plant Mini Kit assisted in improving the

quality of DNA isolated and this DNA was used in downstream applications (**Figure 4.4A** shows intact genomic DNA). The concentration of DNA isolated using the kit was always greater than 15 µg and the $A_{260/280}$ ratio which indicates the level of protein contamination was always between 1.8 and 2.0. According to Sambrook and Russel (2001), the 1.8 to 2.0 UV absorbance ratio for protein contamination represents the best purity showing that nucleic acids are clean and of a high quality. The RNA was also of superior quality represented by a sharp 28S and 18S ribosomal RNA bands (**Figure 4.4B**)**.** Good quality DNA and RNA is essential for the detection of transgenes using hybridisation and PCR techniques to confirm integration and expression of genes.

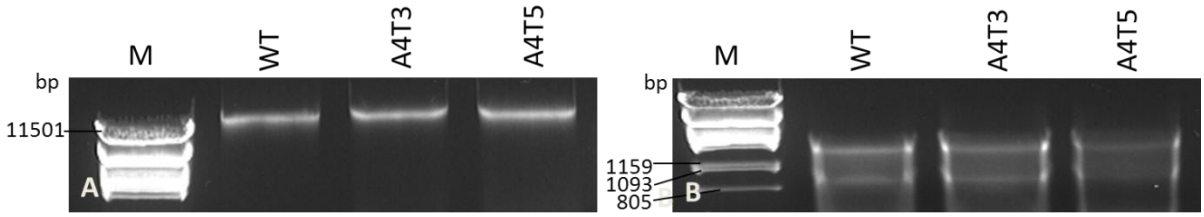

Figure 4.4 Genomic DNA (A) and RNA (B) isolated from *Salvia runcinata* using the Invisorb® Spin Plant Mini Kit and CTAB method respectively.

4.3.3.2. Detection of *rol* and *ags* genes in transgenic tissue through PCR

The PCR analysis of the two putative hairy lines (A4T3 and A4T5) confirmed positively the presence of the *rol A*, *B*, *C* (T_L-DNA) and *ags* (T_R-DNA) genes with a 300, 400, 600 and 1600-bp visible bands for each gene respectively (**Figure 4.5**). Studies have indicated that not all the genes may be detectable from transgenic lines with the possibility of either the integration of the T_L-DNA or T_R-DNA or both (Batra *et al.,* 2004; Guivarch *et al.,* 1999), but we have found the integration of both in the two lines analysed. All control (untransformed roots (WT) and the H_2O (blank)) were negative.

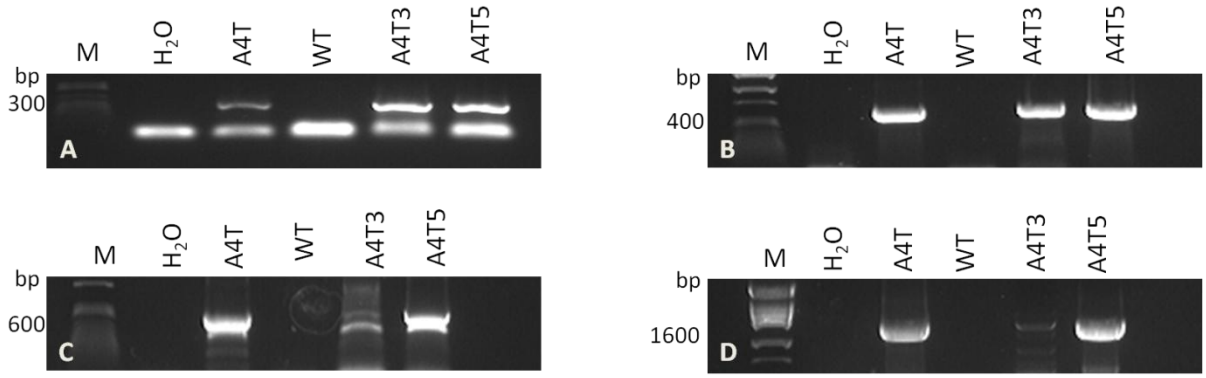

Figure 4.5 A PCR segment of (A) *rol* A resolved at 300 bp; (B) *rol* B resolved at 400 bp; (C) *rol* C resolved at 600 bp and (D) the *ags* gene resolved at 1.6 bp was positively detected for each gene analysed.

Through the RT-PCR assay, the expression of the *rol A, B, C* and *ags* genes was confirmed in the two transformed lines (A4T3 and A4T5) (**Figure 4.6**). Primers used for *rol B* amplification led to non-specific amplifications hence there is more than one band showing resultant amplicons in plasmid A4T, WT, A4T3 and A4T5. Most importantly, the expected band for this gene was amplified in the plasmid A4T (control), line A4T3 and line A4T5. Expression of *rol A, B* and *C* genes leads to a succession of events and promotes the induction of the hairy root syndrome as the genes act synergistically (White *et al.,* 1985; Schmülling *et al.,* 1988, 1989; Tiwari *et al.,* 2008). Out of the *rol* genes, *rol* B is important in inducing the production of hairy roots and it is essential for this role whereas *rol* C is often used to produce plants with a dwarfed phenotype (Giri and Narasu, 2000). In this study, the presence of the *rol* genes provided evidence of successful transgene introduction mediated through *A. rhizogenes* transformation, validating the protocol established here.

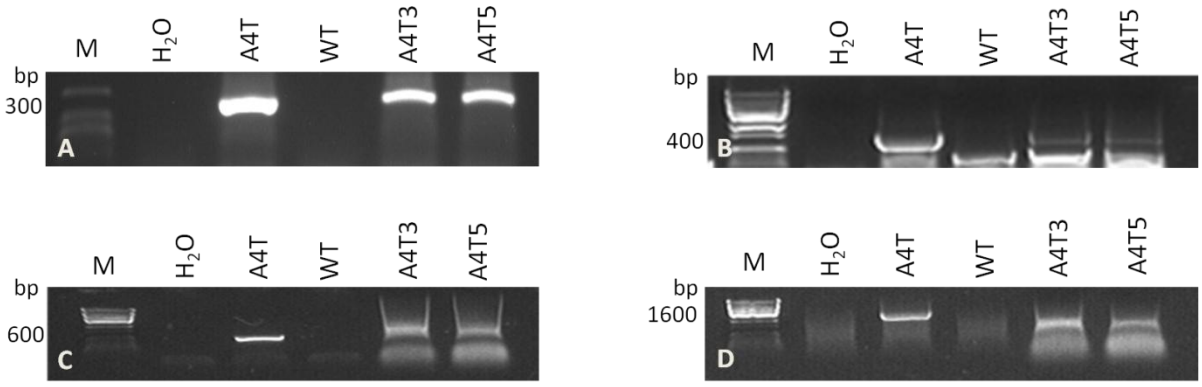

Figure 4.6 A RT-PCR segment of (A) *rol* A resolved at 300 bp; (B) *rol* B resolved at 400 bp; (C) *rol* C resolved at 600 bp and (D) the *ags* gene resolved at 1.6 bp was positively detected confirming expression of each gene analysed.

4.3.5. Detection of *rol* genes in transgenic tissue through Southern blot analysis

The integration of the T-DNA of Ri plasmid was further confirmed by Southern blot analysis after digestion of genomic DNA. The digestion with 70 U of *Eco*R1 over three days facilitated complete digestion of the 20 µg DNA and this was shown on the gel by a constant smear with visible small fragments (**Figure 4.7A**).

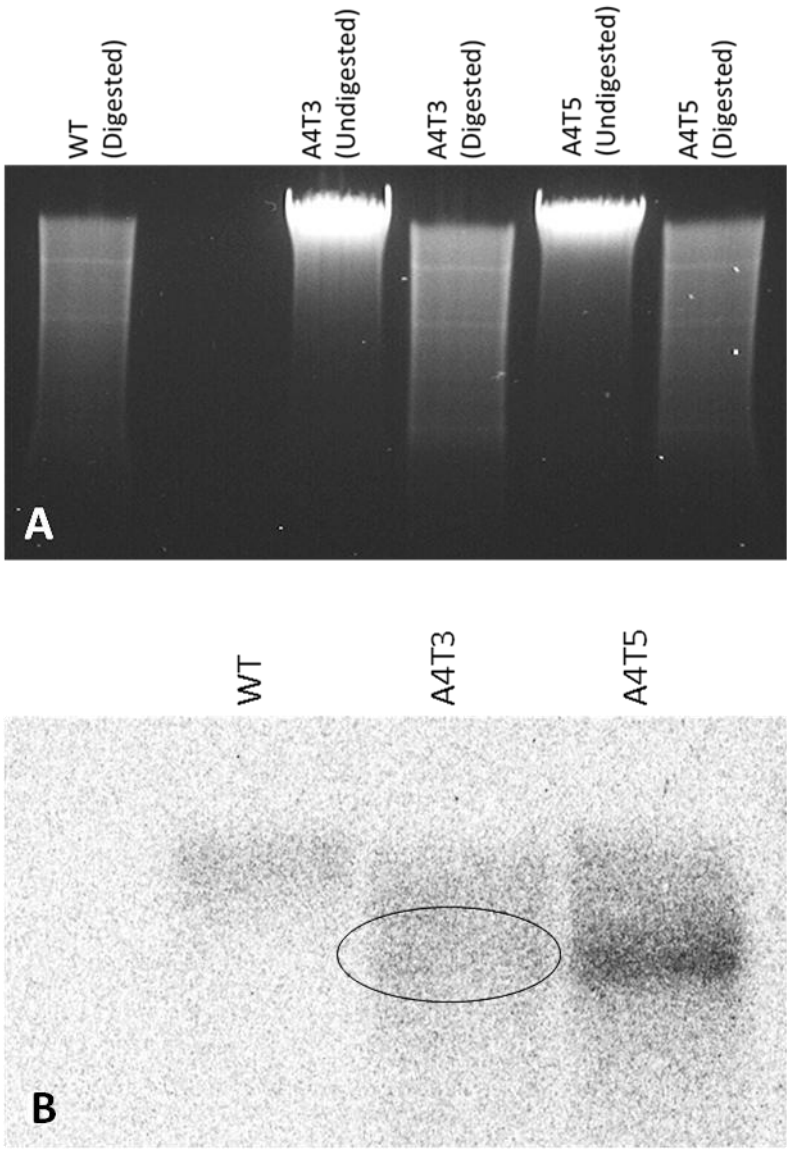

Figure 4.7 (A) Genomic DNA digested for 72 h with *Eco*R1 restriction digests of lines analysed using Southern blot for the integration of *rol* A gene; (B) Detection of *rol* A gene from *A. rhizogenes* T-DNA in the transformed regenerants by Southern blot hybridization analysis.

Although the detection signal of clone A4T3 was very weak, positive hybridisation was still sufficient to confirm the stable integration of the *rol* A gene (**Figure 4.7B**). For the A4T3 clone a distinct band was visible showing stable integration of the *rol* A gene. This provided further evidence of genetic transformation. Each transformation event is distinct from the other due to different integration sites and copy numbers of

Ri T-DNA (Saxena *et al.,* 2007). This was the first time that *S. runcinata* was transformed.

4.4. Conclusion

Hairy root transformation was easily achieved through use of both A4T and LBA 9402 strains but only A4T clones were stably transformed. This protocol described in this chapter may easily be adopted for other *Salvia* species.

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Chapter 5

Phytochemistry and pharmacology of *Salvia runcinata* L.f.

5.1. Introduction

Resistance to antibacterial, antiviral, antiparasitic and antifungal products is becoming a major problem worldwide (Ghannoum and Rice, 1999; Kumar *et al*., 2006). The resistance to these drugs by infectious microorganisms has led to the emergence of new and re-emergence of old infectious diseases (Hemaiswarya *et al*., 2008). In spite of the improvement in the understanding of the growth patterns and control of these pathogens, nearly all the diseases affecting millions of people in southern Africa and the world as a whole are still as a result of microorganisms (Kamatou *et a*l., 2007). There is thus a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action.

Ethnopharmacologists, botanists, microbiologists, and natural-products chemists have been prospecting the earth for phytochemicals and 'leads' which could be developed for the treatment of these pathogenic microorganisms causing infections (Cowan, 1999). Following the long use of plants by traditional healers and indigenous people, natural products derived from plants and natural plant compounds are the focus of some biotechnological companies as they have been discovered to offer unlimited potential leads for new antimicrobial and antibiofilm drugs (Cos *et al*., 2006; Kamatou *et al.*, 2007; Kuźma *et al*., 2007). These plants have been found *in vitro* to possess a wide variety of secondary compounds such as tannins, terpenoids, alkaloids, and flavonoids which are believed to have antimicrobial properties since plants exploit them for their defence (Drewes *et al.,* 2006). *Salvia* species have also been found to be potential candidates in regards to antimicrobial agents as they have been extensively studied throughout the world and reported to have various antimicrobial activities (Ulubelen, 2003; Fisher, 2005;

Kamatou, 2006; Topçu *et al*., 2008). Some of the compounds in *Salvia species* have been isolated and their structures elucidated, nevertheless, many of the compounds are still scientifically challenging.

5.1.1. Flavonoids in *Salvia* species

The flavonoids in the flowers of *Salvia* species are primarily there to provide colours that are attractive to their pollinators, and in leaves, these compounds are believed to promote physiological survival, protecting the plants from fungal and UV-radiation (Cushine and Lamb, 2005). Flavonoids found in plants generally have strong antioxidant activity for scavenging free radicals which are involved in cell damage and tumour production (Lu and Foo, 2002). These compounds exhibit significant inhibitory activity against bacteria and fungi. 6-Hydroxyflavones are the flavonoids that characterise the species of *Salvia* and they include a variety of 6-hydroxylated apigenin and luteolin derivatives with cirsimaritin (Lu and Foo, 2002; Kamatou, 2006). Salvigenin has been isolated in *Salvia radula* and it exhibited moderate activity when tested against the MCF-7 (breast cancer cell line) cells (Kamatou *et al*., 2008).

5.1.2. Triterpenoids in *Salvia* species

Triterpenoids are fairly high in most *Salvia* species but much attention has been paid to the diterpenoids, phenolics and volatile compounds (monoterpenoids) (Topçu, 2006). The triterpenoid compounds exhibit a wide variety of biological activities including, to mention a few, antioxidant (Topçu *et al*., 2007), antiulcer, antifertility, antiinflammatory (Mitaine-Offer *et al*., 2002). Two urserne-type triterpenoids that were isolated from the hairy roots of *Salvia sclarea* and studied by Marwani *et al.* (1997) showed that the ursonic acid compounds were active against *Escherichia coli* and *Bacillus subtilis*. Nine triterpenoids were isolated from *Salvia chinensis* and were tested for their antiproliferative effects using human leukemia HL-60 cells. The nine compounds were all found to have various abilities to inhibit the growth of these cells with one compound showing a GI_{50} value of 7.77 μM denoting high potency (Wang *et al*., 2009).

5.1.3. Monoterpenes in *Salvia* species

Monoterpenes are the major components of many essential oils and are usually obtained by steam distillation or solvent extraction of leaves and may be more infrequently distilled from the roots (Banthorpe *et al.,* 1971). These compounds have economic importance as flavors and perfumes hence many *Salvia* species like *Salvia stenophylla* provide important components for cosmeceutical products (Musarurwa *et al.,* 2011). Monoterpenes have been found to form the major components in the essential oils of *Salvia miltiorrhiza* (Liang *et al.,* 2009), *Salvia cryptantha* and *Salvia multicaulis* (Tepe *et al.,* 2004), *Salvia stenophylla* (Viljoen *et al.,* 2006; Musarurwa *et al.,* 2011), *Salvia muirii* (Kamatou *et al.,* 2006), *Salvia dolomitica* and *Salvia chamelaeagnea* (Kamatou, 2006) and many other *Salvia* species, and are implicated in the antimicrobial activities of these plants.

5.1.4. Diterpenoids in *Salvia* species

Diterpenoids exhibit a vast array of biological activities including bactericidal, insecticidal, antiviral, and several others in *Salvia* species (Al-Hazimi and Miana, 1994; Rodriguez-Hahn *et al.,* 1995; Kabouche and Kabouche, 2008). Most of these compounds are considered to be resins, the material that remains after the plant extracts have been steam distilled (Cseke *et al*., 2006). Carnosol, rosmadial, ursolic acid and carnosic acid have been isolated from *Salvia africana-lutea* L. using an ethanolic solvent with carnosic acid showing higher antimycobacterial activity with an minimum inhibitory concentration (MIC) of 28 µM (Hussein *et al.,* 2007). Carnosic acid and carnosol have also received much attention in food sciences and medicine because of their potent antioxidant properties.

In this study, the major objective was to investigate the antibacterial and antifungal activities of *Salvia runcinata* shoot tissue and hairy root organ cultures. These were tested against two Gram-negative bacterial strains *Escherichia coli* (ATCC 11775) and *Klebsiella pneumoniae* (ATCC 13883), two Gram-positive bacterial strains *Bacillus subtilis* (ATCC 6051) and *Staphylococcus aureus* (ATCC 12600) and lastly, two *Fusarium* species, *Fusarium proliferatum* (MRC 6908) and *Fusarium*

subglutinans (MRC 0115). Of the three related taxa (*S. stenophylla*, *S. repens* and *S. runcinata*) tested for antioxidant, antimalarial and antiinflamatory by Kamatou (2005) and co-workers, *S. runcinata* exhibited the most favourable activity. The drive for the study was therefore to establish whether tissue culture and *Agrobacterium*-mediated transformation have any effect on the biological activity and chemistry of *S. runcinata*.

5.2. Materials and Methods

5.2.1. Preparation of plant extracts

The two hairy root clones (A4T3 and A4T5), roots excised from tissue culture plants and foliage tissues (generated from 2 month old *in vitro* and *ex vitro* plants) were pulverized with liquid nitrogen using a pestle and mortar. Two separate extraction solvents were used for the study: acetone or methanol: dichloromethane (MeOH:DCM) (1:1 v/v). The former was shown by Eloff (1998b) to be a very useful solvent owing to its wide extraction capacity and low toxicity towards the test organism. MeOH:DCM was also proven by Ramogola (2009) to be an effective extractant of both polar and non-polar biochemicals and extracts were most potent against test *Fusarium* species. Four grams of tissue was mixed with 4 ml of each extraction solvent and sonicated for an hour at room temperature. The extracts were filtered using Whatman filter paper No. 1. The extraction process was repeated twice to maximise the extracted products. The filtered extracts were evaporated to dryness under vacuum at 40 °C in a rotary evaporator (BUCHI 461) to yield a concentrated residue. Extracts in conical flasks were covered in foil and were stored dry in the dark at 4 °C prior to use. The residues were then resuspended in acetone to give a final concentration of 50 mg ml^{-1}.

5.2.2. Test microorganisms and bacterial growth conditions

All media were autoclaved for all microbial tests. Two Gram-negative bacterial strains: *Escherichia coli* (ATCC 11775) and *Klebsiella pneumoniae* (ATCC 13883), and two Gram-positive bacterial strains: *Bacillus subtilis* (ATCC 6051) and *Staphylococcus aureus* (ATCC 12600) were used as test microorganisms on which activity of the extracts was tested against. Bacterial colonies were grown in liquid Müeller-Hinton (MH) broth (Flika Biochemika, Spain) overnight at 37 °C on an orbital shaker at 200 rpm. The bacterial suspensions were adjusted to 1 X 10^6 colony forming unit (CFU) mL^{-1} using a spectrophotometer at a wavelength of 625 nm. The protocol for the bacterial assay follows in Section 5.2.5.

5.2.3. Fungal inoculum preparation

Two strains of *Fusarium* species: *Fusarium subglutinans* (MRC 0115) and *Fusarium proliferatum* (MRC 6908) were donated by Mrs Lindy Rose from the Stellenbosch University, Plant Pathology Department. The fungal isolates were grown in liquid Armstrong medium (1.1 g KH_2PO_4; 0.4 g $MgSO_4 \cdot 7H_2O$; 1.6 g KCl; 7.27 g KNO_3; 2 parts per million (ppm) $FeSO_4 \cdot 7H_2O$; $ZnSO_4 \cdot 7H_2O$; $MnSO_4 \cdot 2H_2O$; 1.0 mg thiamine-HCl and 0.05 ml polyoxyethylene (20) sorbitan monooleate (Tween® 80; Merck, Germany) in 1 L of distilled water at 25 °C) at 100 rpm for 4-5 days. The spores were then filtered using sterile cheese cloth and the suspension was centrifuged at 3500 relative centrifugal force (rcf) for 10 min to remove the supernatant. The conidia were washed twice with de-ionised distilled water with centrifuging between washes. The pellet of spores was resuspended in 250-500 ml of sterile distilled water. The spore concentration was then determined and adjusted to 2 X 10^6 conidia ml^{-1} by means of haemocytometer. Polyoxyethylene (20) sorbitan monolaurate (Tween® 20; Merck, Germany) surfactant was added to the conidial suspension before use at a rate of three drops L^{-1}. The protocol for the fungal assay follows in Section 5.2.6.

5.2.4. Thin Layer Chromatography (TLC) analysis

Thin layer chromatography was performed using silica plates (0.25 mm silica gel 60 F_{254}; Merck, Germany) to determine the secondary metabolite profile of each extract. Ten microliters of each extract was separated using the TLC and developed using a toluene:ethylacetate:diethylamine (10:20:10 v/v) mobile phase in an ascending one-dimensional mode. The separated bands were examined under 254 nm and 366 nm UV light prior to spraying with anisaldehyde-R stain (465 ml ethanol, 5 ml glacial acetic acid and 13 ml concentrated sulphuric acid). The sprayed TLC plates were incubated immediately at 100 °C for 5 min to develop the stain. The secondary metabolite profiles of the hairy roots clones and foliage tissue were compared after the development of the TLC chromatograms.

5.2.5. Antibacterial activity assay

To screen *S. runcinata* extracts for antibacterial activity, a microdilution technique as described by Eloff (1998a) was used. Plant extracts (50 mg ml^{-1}) were two-fold serially diluted in sterile distilled water down the wells of a 96-well microplate. Positive and negative controls were set-up; an antibiotic streptomycin was utilized as a positive control and the negative controls were sterile distilled water, bacteria-free MH broth and acetone which was also used as a resuspension solvent. The plates were covered with Parafilm 'M'® (American National Can™, USA) and incubated at 37 °C for 18 h. Subsequently the growth of bacteria was detected by the addition of 40 µl of 10 mg ml^{-1} *p*-iodonitrotetrazolium chloride (INT) (Sigma, USA) into each well after which the plates were returned to the 37 °C incubator for 1 h prior to data collection. The lowest concentration of the test sample in which no growth occurred was defined as the MIC (Eloff 1998a).

5.2.6. Antifungal activity assay

To screen the extracts for antifungal activity, a microdilution technique as described by Thembo *et al.* (2010) was adopted with some modifications. Plant extracts (50 mg ml^{-1}) were two-fold serially diluted as described in Section 5.2.5. Positive and

negative controls were set-up; a clinical fungicide Amphotericin B (Sigma, USA) (active at 1 µg ml^{-1}) and an agricultural imidazole fungicide Prochloraz (prochloraz as an active ingredient at 100 µg ml^{-1}) (Chemsky, Shanghai) were utilized as positive controls; predistilled water, Armstrong medium and acetone were negative controls. The plates were covered with Parafilm and incubated at room temperature for 5 days. After the first 24 h of incubation, 40 µl of 10 mg ml^{-1} INT was added in each well as an indicator of fungal growth. The plates were returned to room temperature incubation for the remaining 96 h with 24 hourly intervals for the recording of growth. The results were recorded in terms of the MIC.

For all the microdilution bioassays (antibacterial and antifungal tests), the experiments were done in triplicate. Since the microdilution assay is a susceptibility test, no statistical analysis was performed.

5.2.7. Gas chromatography-mass spectrometry (GC-MS) analysis

5.2.7.1 Volatiles (Headspace- solid-phase microextraction (SPME) GC-MS)

To make a comparison of the volatile compounds of *Salvia runcinata* plants grown in two different environmental conditions, 400 mg each of leaves from two month old *in vitro* and *ex vitro* plant material was harvested, immediately placed in 20 ml head-space vials sealed with an aluminium-coated silicone rubber septum which is easily pierced with the needle of the 'GC-MS' instrument. The samples were sent for GC-MS analysis and the protocol from Musarurwa *et al.* (2010) was adopted. The HS-SPME of the leaflets was performed with Supelco SPME fibres (DVB/ Carboxen/PDMS, StableFlex (Supelco). Volatiles were extracted at 80 °C for 15 min. The gas chromatography was performed with a Waters GCT Premier AS 2000 instrument coupled to a mass spectrometer, equipped with a HP-5 column (30 m, 0.25 mm i.d., 0.25 µm film thickness). Temperatures were set at 250 °C for both the injection (split injection ratio of 1:5) and the ion source temperature. Helium was used as the carrier gas (1 ml min^{-1}). The temperature ramp regime was initiated by heating at 40 °C for 5 min, followed by an oven ramp to 150 °C at 5 °C min^{-1}; and a second ramp of 10 °C min^{-1} up until 280 °C. A mass scanning range of 40–550 m/z (perfluorotri-N-butylamine as mass reference) was employed and mass spectra were

recorded at 2 scans s^{-1}. The data were analysed using MassLynx™ software and the National Institute of Standards and Technology (NIST) library was used to identify the compounds. The available authentic standards in **Table 5.1** were used for confirmation of identified compounds.

Table 5.1 Authentic standards used for headspace-SPME confirmation of identified compounds.

Standard name	**Manufacturer**
Camphene	ALDRICH®
(IR)-(+)-Camphor	ALDRICH®
β-Caryophyllene	SΛFC™
Caffeic acid	SIGMA®
Camphene	ALDRICH®
1,4 Cineole	Fluka
Caryophyllene oxide	SΛFC™
(IR)-(+)-α-Pinene	SΛFC™
(-)-α-Bisabolol	ALDRICH®
3-Carene	Fluka
R-(+)-Limonene	Fluka
α-Myrcene	Fluka
(-)-α-Thujone	Fluka

5.2.7.2. Non-Volatiles

Twenty microlitres of each extract were vacuum dried in a SpeedVac® Plus SC 110A (Salvant, New Jersey). Hundred microliters of MeOH was used to clean the extracts by washing the extracts twice, using the SpeedVac® to evaporate the MeOH each time. The Glassop *et al.* (2007) protocol was used for chemical derivatisation of extracts rendering them volatile. A 20 µl aliquot each of 0.2 mg ml^{-1} ribitol was added to the MetOH on the second wash in all the extracts and used as an internal standard. The dried residue of the extracts was resuspended in 60 µl methoxyamine hydrochloride (40 µl from 20 mg ml^{-1} in pyridine) and the samples were incubated at

37 °C for two h at 300 rpm with constant shaking. The samples were then derivatised using trimethlychlorosilane:hexamethyldisilazane:pyridine (TMS) (1:3:9 v/v). The TMS-derivatised samples were then incubated at 37 °C for 30 min and were subsequently sent for GC-MS analysis.

One microlitre of the derivatised sample was injected with a split-less injection for analysis into the GC-MS system with a flow rate of 1 ml min^{-1}. The GC-MS system consisted of an AS 2000 autosampler, trace GC and a quadropole trace MS (Thermofinnigan). The gas chromatography was conducted on a 30 m Rtx®-5Sil MS column (Restek) with an Integra Guard with an inner diameter of 0.25 mm and 0.25 mm film thickness. The injection temperature was 230 °C and the ion source temperature was 200 °C. The following temperature programme was used: 5 min at 70 °C, followed by the first ramp of 1 °C min^{-1} to 76 °C and a second ramp of 4 °C min^{-1} to 320 °C. Before the injection of the next sample, the temperature of the system was then equilibrated to 70 °C. The mass spectra were recorded at 2 scans s^{-1} with the scanning range of 30-600 *m/z*. The data were analysed using an Automated mass spectral deconvolution and identification system (AMDIS) programme which was linked to the National Institute of Standards and Technology (NIST) library to identify and quantify the metabolites in all the extracts.

5.3. Results and Discussion

5.3.1. TLC analysis

As expected, acetone and MeOH:DCM extracted more compounds in the foliage material than the transgenic roots. As in traditional medicines, foliage is generally favoured for preparing herbal remedies from African sages (Kamatou, 2009) and roots are rarely consumed for medical purposes and if so, usually in higher doses (Watt and Breyer-Brandwijk, 1962). Nevertheless, some of the compounds were only present in the transgenic root extracts and could not be detected in the foliage extracts (**Figure 5.1**). This inter-clonal variation is a fundamental characteristic of

secondary metabolites as they may be frequently limited to particular organs, cells and even tissues within that organ (Rhodes, 1994).

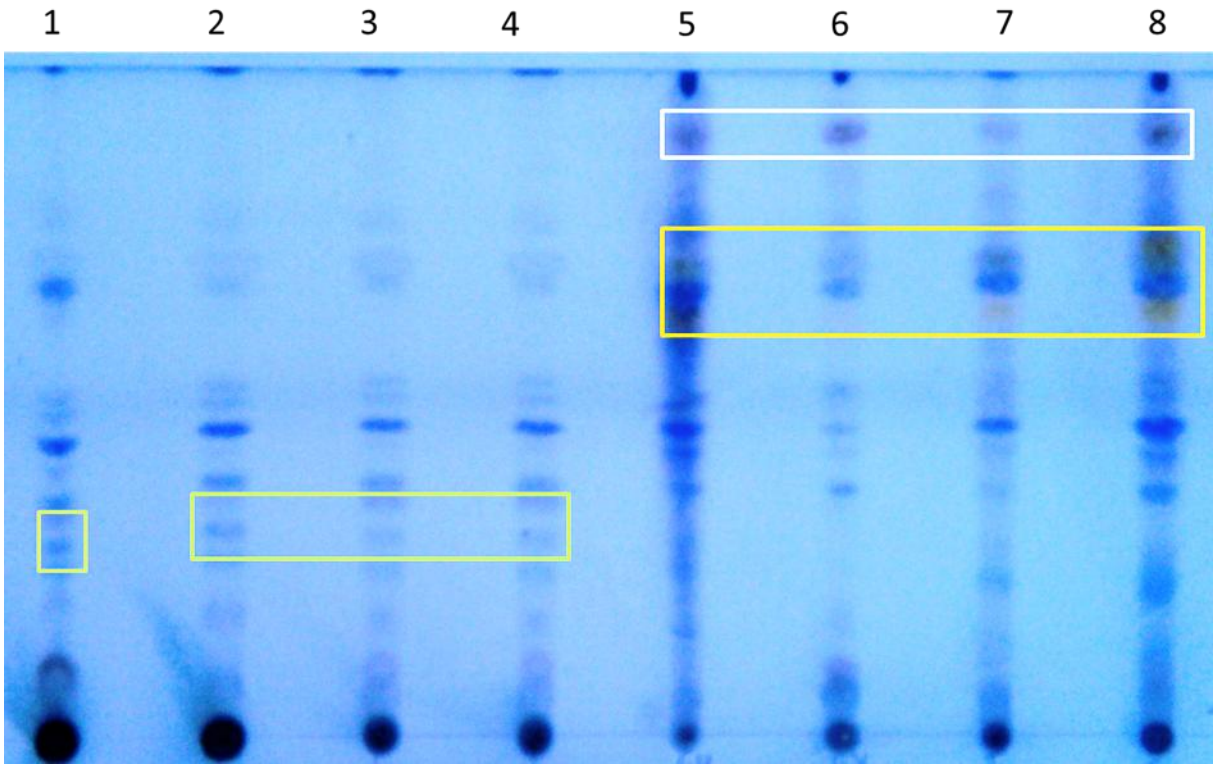

Figure 5.1 Chromatogram of transgenic roots and leaf extracts from *in vitro* and *ex vitro Salvia runcinata* material sprayed with anisaldehyde-R stain. (1) Acetone extract of A4T3 line, (2) MetOH:DCM extract of A4T3 line, (3) Acetone extract of A4T5 line, (4) MetOH:DCM extract of A4T5 line, (5) Acetone extract of glasshouse grown plants (GH), 6) MetOH:DCM extract of GH, (7) Acetone extract of tissue cultured plants (TC), (8) MetOH:DCM extract of TC. All bands indicated with the rectangles represent compounds that were present in non-transgenic extracts but not present in transgenic extracts and vise versa.

5.3.2. Antibacterial activity assay

The bacteria used in the study were chosen as they are important pathogens in nosocomial infections. *Staphylococcus aureus* is one of the notorious pathogens as it readily changes its epidemiology, becoming resistant to antibiotic drugs

(Grundmann *et al.,* 2006). The acetone and MetOH:DCM extracts of *in vitro* plant cultures exhibited good to moderate antibacterial activity in all the bacteria tested with MIC values ranging from 0.39 to 0.78 mg ml^{-1} (**Figure 5.2**). As the antibacterial action ranged between 0.19 to 1.56 mg l^{-1} for *ex vitro* and *in vitro* plant cultures, this suggested that micropropagated plants maintain their secondary metabolite integrity and bioactivity even under new controlled micro-environmental conditions. Interestingly, it was noted that the acetone extracts of both the *in vitro* and *ex vitro* plant extracts were more active against the Gram-positive bacteria (0.19 to 0.39 mg ml^{-1}). The work of Moujir *et al*. (1993), Baricevic and Bartol (2000), Ulubelen (2003), Mayekiso *et al.* (2008) support this as plants extracts had better inhibition on the growth of Gram-positive bacteria. The general lower activity of extracts against Gram-negative bacteria is mainly ascribed to the presence of the outer hydrophobic phospholipidic membrane in these bacteria, which is almost impermeable to lipophilic compounds (Delamare *et al.,* 2007). The efficiency of acetone as the best extraction solvent was shown by Eloff (1998b) as it dissolved many hydrophylic and lipophilic components from *Anthocleista grandiflora* and *Combretum erythrophyllum,* hence the high activity of acetone extracts in this study.

The hairy roots extracts had poor activity and this may imply that the compounds that have the antibacterial activities are not produced in *Salvia runcinata* roots as the secondary metabolites of hairy roots are often limited to those normally produced in roots (Sévon and Oksman-Caldentey, 2002). Stress also plays a major role in regulating secondary metabolism. It is thus likely that application of stress *in vitro* may elevate the production of secondary metabolites, contributing to improving pharmacological activity (Sharma *et al.,* 2011). The chemical composition of plants may be influenced by seasonal changes and even the number of hours plants receive sunlight may influence the phytochemistry therefore bioactivity (Kamatou *et al.,* 2008). In this study, micropropagation assisted with producing plants that are uniform with a consistent constituency of chemicals with better antibacterial activity compared to those analysed by Kamatou *et al*. (2008) growing in nature. This validated the use of a tissue culture approach.

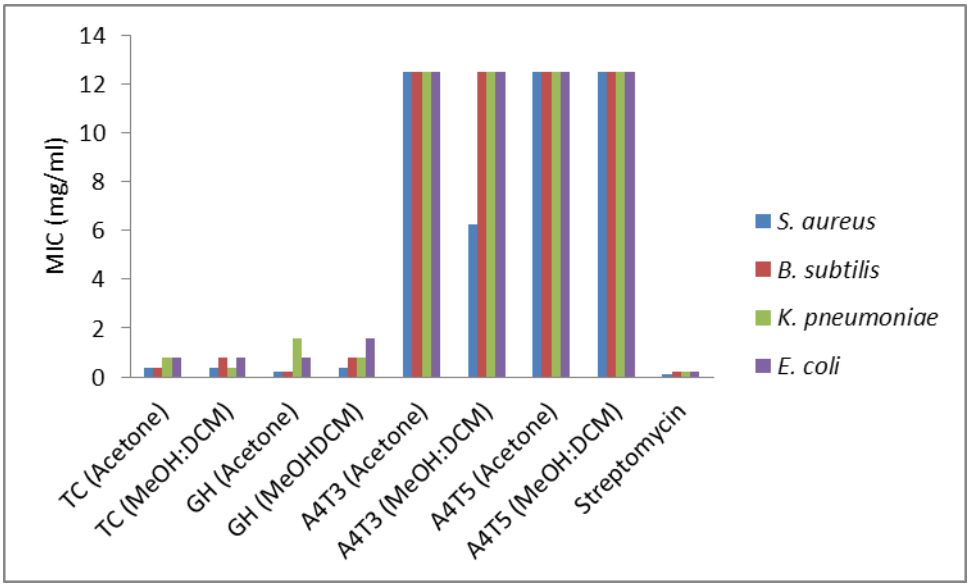

Figure 5.2 The antibacterial activity of *Salvia runcinata* tissue and hairy root organ cultures against various pathogens. Significant values are those below 1 mg ml^{-1} (Van Vuuren, 2008).

5.3.3. Antifungal activity assays

Fusarium infections are renowned for wide losses in South African maize producing areas and other agricultural sectors (Viljoen *et al.,* 1997). *Fusarium subglutinans* and *Fusarium proliferatum* are among the frequently isolated *Fusarium* species from maize in temperate climates. *F. subglutinans* is not only found in maize, it has been associated with many hosts including pines (Viljoen *et al.,* 1997). *F. proliferatum* on the other hand has been shown to cause maize ear rot and produces fumonisins and other mycotoxins (Velluti *et al.,* 2000). Fumonisins in maize pose a carcinogenic risk to humans (Rheeder *et al.,* 2002) and plants. *S. runcinata* extracts were thus investigated for their activity against these fungi. The tissue culture MetOH:DCM extract was found to be the most potent extract against both *F. proliferatum* and *F. subglutinans* with the MIC ranging from 0.08 to 0.16 mg ml^{-1} over a period of 120 h (**Figure 5.3 and 5.4**).

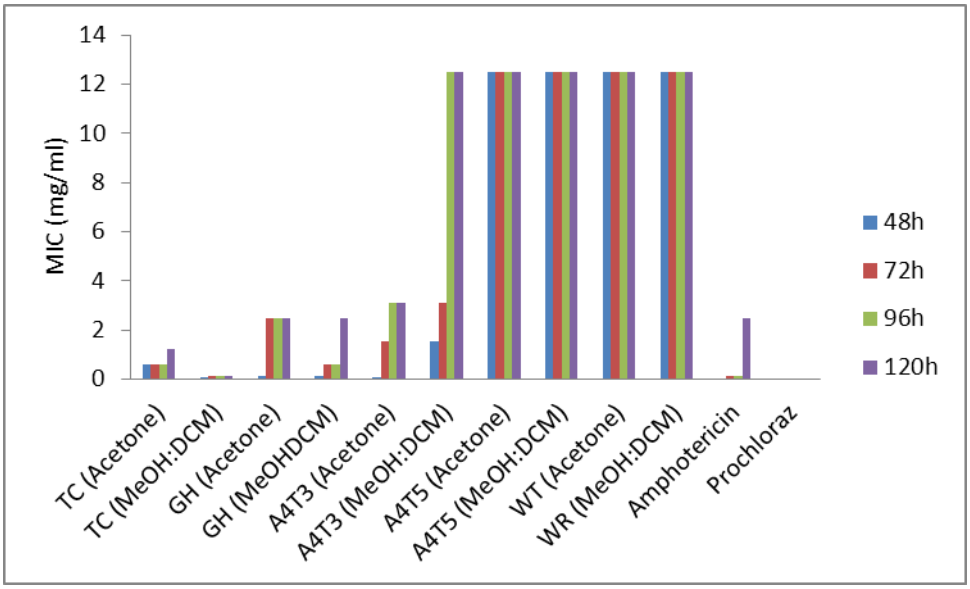

Figure 5.3 Antifungal activity of *Salvia runcinata* tissue and hairy root organ cultures against *Fusarium proliferatum*. Significant values are those below 1 mg ml^{-1} (Van Vuuren, 2008).

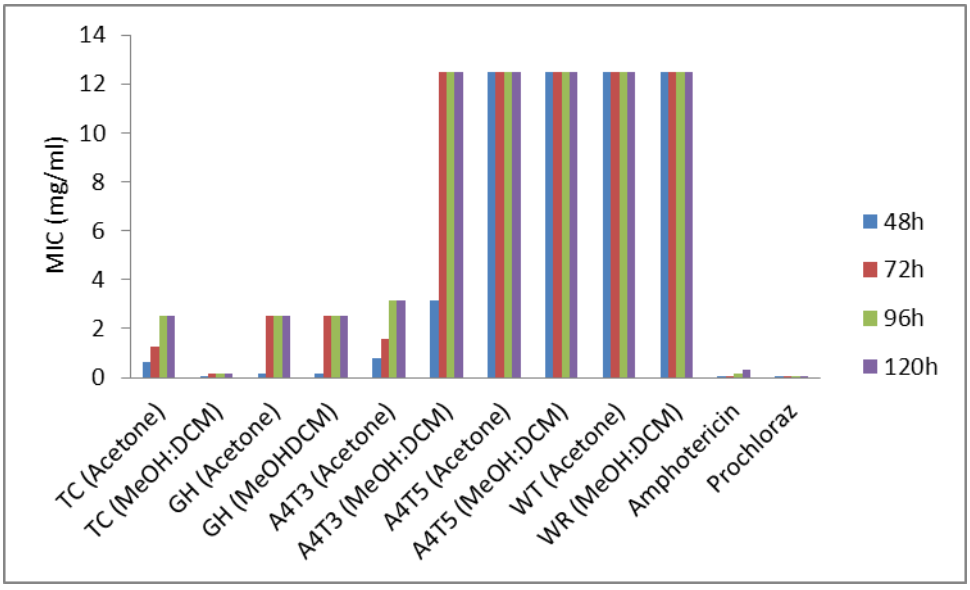

Figure 5.4 Antifungal activity of *Salvia runcinata* tissue and hairy root organ cultures against *Fusarium subglutinans*. Significant values are those below 1 mg ml^{-1} (Van Vuuren, 2008).

The tissue culture MetOH:DCM extract were fungistatic maintaining the inhibition effect on the growth of the two fungal pathogens over a period of 120 h. The activity of this *in vitro* shoot extract was comparable to those of positive controls and therefore promises to be a good natural antifungal agent. The hairy root extracts did not show any activity against the fungal pathogens, as was the case with the bacteria. This is in contrast to the data generated for the hairy root cultures of *S. africana-lutea* which had excellent activity against *Fusarium* (Ramogola, 2009).

5.3.4. GC-MS analysis

5.3.4.1. Volatiles (Headspace-SPME GC-MS)

The compounds identified from the *in vitro* and *ex vitro* leaves of *S. runcinata* are listed in **Table 5.2** in order of their elution time from the HP-5 column. A total of 26 compounds were putatively identified. The *in vitro* and *ex vitro* leaves of *S. runcinata* were mainly composed of sesquiterpenes (53%), monoterpenes (42%) and some fatty alcohols (0.04%). Oxygen-containing sesquiterpenes were also found to be dominating in the *S. runcinata* essential oil fraction when Kamatou and his co-workers (2008) hydro-distilled it. Caryophyllene was the most abundant component in both the *in vitro* and *ex vitro* leaves (25.9% and 22.65% respectively), followed by β-bisabolone (18.29% in *in vitro* plants), (+)-ledene (10.55% in *in vitro* plants) and α-pinene (8.47% in *in vitro* plants) (**Table 5.2**). The α-bisabolol level was at 3.17% in *in vitro* plants as compared to the chemotype identified by Kamatou and his co-workers (2008) from the wild which had over 65% of this compound. The compounds; 1.8-cineole (eucalyptol) and borneole, which are also reported to be characteristic constituents of many *Salvia* volatile components, were found in relatively small quantities in *S. runcinata*. Tepe *et al.* (2005) also detected low quantities of these two volatiles. The constituents of aromatic oils are highly dependent on environmental conditions, stress factors and also the age of the plant. The change in the secondary metabolite profile is directly correlated to the biological activities of the plant-derived extracts.

Table 5.2 Relative percentage composition of the volatile compounds of *Salvia runcinata* in *in vitro* and *ex vitro* leaves.

Components	**[1]t_R (min)**	***Ex vitro* plants (%)**	**t_R (min)**	***In vitro* plants (%)**
α-Pinene*	4.58	1.80	4.61	**8.47**
β-Pinene	6.77	0.89	6.78	**3.20**
3-Carene*	7.96	**5.29**	7.94	**2.36**
(+)-4-Carene	8.87	0.14	8.89	0.20
3-Thujene	8.41	0.29	8.41	0.22
β-Myrcene	8.62	0.20	8.63	0.40
β-Phellandrene	9.78	0.77	9.80	0.59
D-Limonene*	9.50	0.77	9.52	0.59
Eucalyptol	9.99	0.68	10.00	0.93
3-Octanol	-	-	16.08	0.29
Isoledene	17.58	0.39	17.60	0.21
α-Gurjunene	19.22	**6.90**	19.19	0.19
(-)-Aristolene	20.16	0.53	20.16	0.09
Caryophyllene	20.97	**22.65**	21.01	**25.90**
β-Humulene	21.17	**8.91**	21.17	**3.01**
Allo-Aromadendrene	22.05	**2.11**	22.06	0.45
α-Caryophyllene	22.66	**5.75**	22.69	**7.63**
Borneol	23.55	0.13	23.55	0.54

Ledol	28.23	**10.96**	-	-
Globulol	29.82	**3.88**	29.82	0.05
(+)-Ledene	30.57	**1.58**	23.29	**10.55**
Camphor*	-	-	19.00	0.19
β-Bisabolone	24.11	**5.91**	32.17	**18.29**
Caryophyllene oxide*	29.12	0.10	29.13	0.29
α-Bisabolol*	32.16	1.40	32.17	**3.17**
Lanceol, cis	34.90	1.23	34.90	1.33

[1] t_R - retention time

[1] The components of *Salvia runcinata* volatiles identified in high amounts (> 2 %) are represented in **BOLD** font. * All the chemicals marked with an asterisk were those confirmed using authentic standards.

As in the study by Musarurwa *et al.* (2010), micropropagation in this study did not significantly affect the volatile compound profile of *S. runcinata* (see **Figure A, Appendix A**[2]). This is important for the commercial application of this technique in the production of *Salvia runcinata* and many other *Salvia* species as it does not have a detrimental effect on the chemical quality of plants. This micropropagation regime would thus be useful for mass generation of *S. runcinata* plants which are chemically superior in their production of epi-α-bisabolol (identified by Kamatou *et al.,* 2008).

5.3.4.2. Non-Volatiles

The majority of the compounds that were identified in *S. runcinata* using GC-MS were sugars, amino acids, organic acids, a few polyphenolic compounds including some phytosterols (**Figure 5.5**). It must be noted that all the compounds identified in **Figure 5.5** were the ones with the closest library match (between 85-100 %). It is assumed that the chemical derivatisation process that was used had an effect on the result of metabolites elucidated as it was originally developed for sugars (Glassop *et al*., 2007). However, the fact that plants naturally carry a lot of primary metabolites which they need for energy for their metabolic processes is not disregarded. Secondary metabolites are mainly produced by plants under stress conditions and for defence (Rhodes, 1994; Taiz and Zeiger, 2006; Verpoorte and Memelink, 2002). Hence under favourable conditions when the plant is subjected to little or no stress, it produces less secondary metabolites. Under micropropagation conditions, the production of secondary metabolites is mainly dependent on the constituents of the different culture media, the light regime and temperature conditions. It is also interesting to note that in a medium defined for optimum growth, secondary metabolite production only occurs later when the primary nutritional constituents are getting depleted (Kim *et al*., 2002).

In this study, plant tissue culture and *Agrobacterium*-mediated transformation had a noteworthy effect on the phytochemistry of *S. runcinata*. An abundance of sucrose, galactose, sorbose and fructose was clearly distinct in the hairy root extracts whereas leaf extracts had lower levels (**Figure 5.5**). This may imply that the hairy roots in suspension culture medium accumulated more of the primary metabolites to assist in growth and biomass accumulation. Sorbose which is a monosaccharide used for the commercial production of Vitamin C (an antioxidant) (Hoshino *et al.,* 2003; Sugisawa *et al.,* 2005) was detected in all the organ culture extracts but was more abundant in clone A4T3 (>15 %). Although no clear pattern depicting the differences in the metabolite profile of the different organ cultures could be identified, A4T3 contained a relatively large amount of sugars than any of the other samples. Out of the detected phenolic acids, not surprisingly, caffeic acid was the most abundant compound among the group, followed by shikimic acid. High levels of caffeic acid are to be expected as it is a building block for many other phenolic compounds generally associated with *Salvia* and serves as a precursor compound for downstream production of other derivatives (Kamatou *et al.,* 2010). Rosmarinic acid was only detected in A4T5 clone, this compound is also often found at high levels in *Salvia verticillata* subsp. *verticillata.* Rosmarinic acid has many *in vitro* biological activities including inhibiting HIV-I, antitumor and antihepatitis action, and protection of the liver, inhibiting blood clots and antiinflammation (Tepe *et al.,* 2007). Several phytosterols were identified in the extracts including stigmasterol, α-sitosterol and campesterol that have significant health benefits. These are not physiologically active, are not highly absorbed and precipitate bile acids thus reducing enterohepatic recycling of colon tumor-promoting biliary components which may result in oxidative damage to crypt cells and micro-nuclei accumulation (Wang and Wixon, 1999).

5.4. Conclusion

The search for novel natural products to control either human or crop infections and pests is a booming promising area of research. Natural compounds produced by secondary metabolism of plants are potentially new sources for new types of pharmo-chemicals. Though there were no novel compounds detected in both the plant shoots and hairy root organ cultures of *Salvia runcinata*, micropropagation promoted the production of several metabolites which may be responsible for microbial inhibition. This cultivation strategy is hence an interesting avenue for commercial production of *S. runcinata,* supplying plants with an improved pharmacological activity in this case.

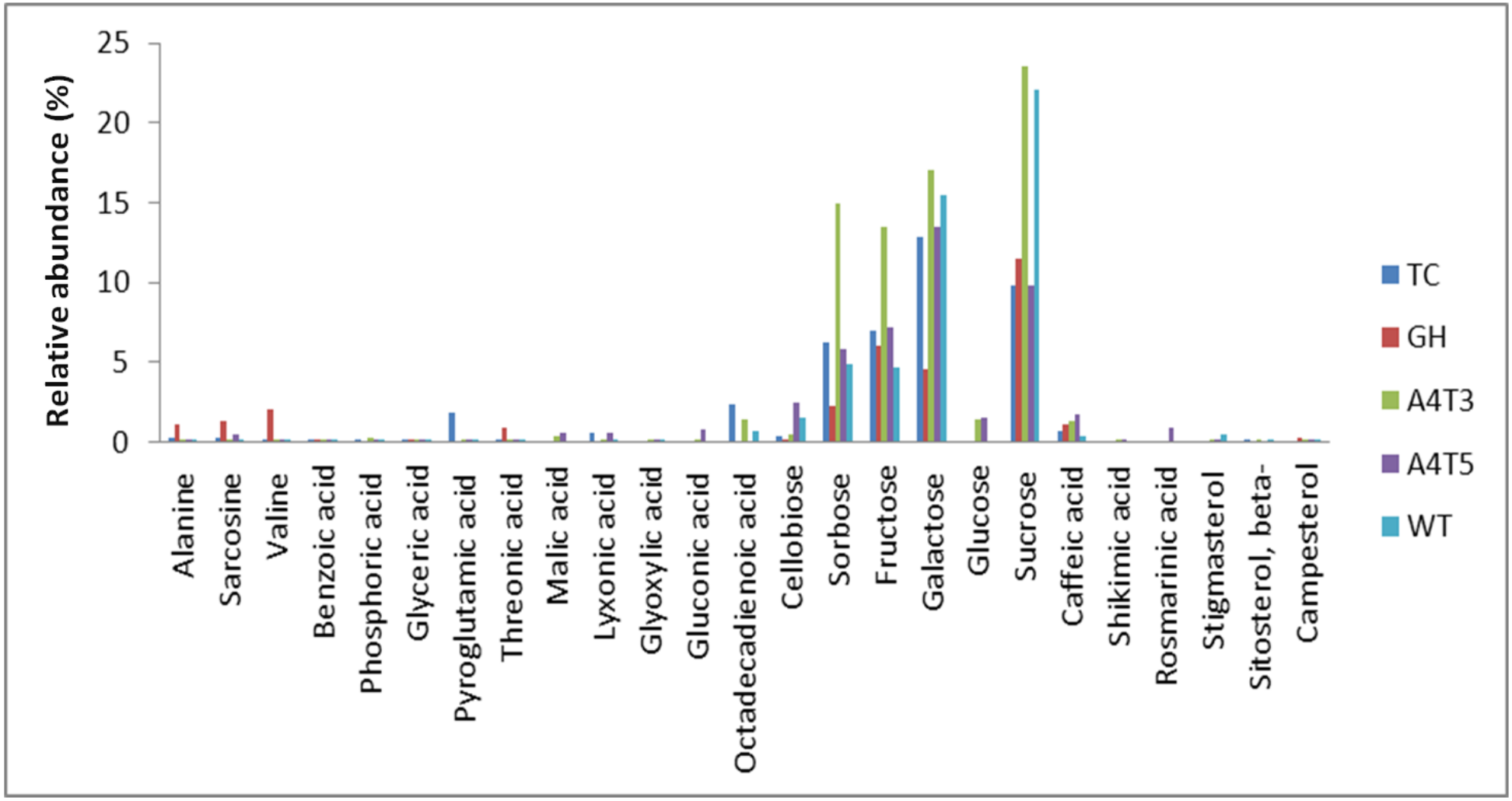

Figure 5.5 Non-volatile compounds of *Salvia runcinata in vitro* and *ex vitro* tissue and hairy root cultures. Results expressed in relative percentage abundance.

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Chapter 6

General conclusion

The successful establishment of an efficient *in vitro* plant culture protocol for *Salvia runcinata* adds to a short list of available micropropagation protocols for African *Salvia* species (namely, *Salvia chamelaeagnea*, *Salvia africana-lutea* and *Salvia stenophylla*). This protocol (depicted below; **Figure 6.1**) is easy to establish, renewable and has potential to generate over an amount of 46656 plants over six months, benefiting aims of commercialisation. This may contribute to the production of a large quality stock of *S. runcinata* for mass generation of a commercial chemotype avoiding problems pertaining to inter- and intra-clonal variation.

The collective data obtained on phytochemical and pharmacological analysis of *S. runcinata* support the use of the biotechnological techniques such as clonal-culture and *Agrobacterium*-mediated transformation in better enhancing the metabolomic profile and bioactivity of *S. runcinata.* This is evidenced by the noted significant improvement in the antibacterial activity of the tissue culture extracts of *S. runcinata* plants. There is a continued economic demand for a wide range of secondary metabolites, particularly in the food and pharmaceutical industries. Successful scaling up of these compounds such as rosmarinic acid, epi-α-bisabolol and other important metabolites in *S. runcinata* through tissue culture and transformation techniques can therefore be adopted for most commercially important medicinal plants to reduce or eliminate the need to cultivate the source plants under variable climatic conditions or, to conduct complex and expensive organic synthensis.

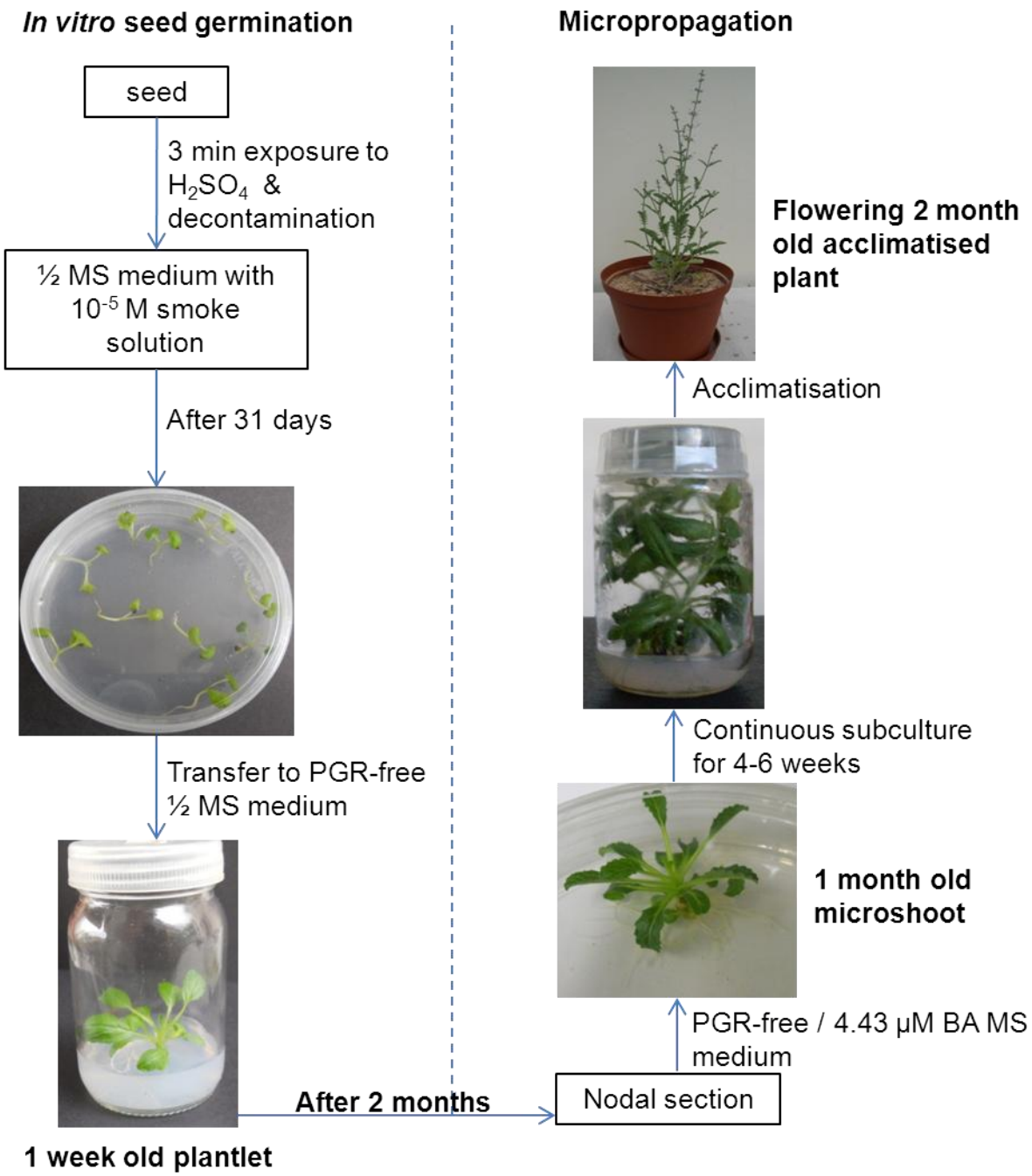

Figure 6.1 Optimal protocol developed for micropropagation of *S. runcinata*.

A better understanding and elucidation, therefore, of the active metabolites in *S. runcinata* and the pathways regulating the biosynthesis of these compounds may eventually provide long-lasting solutions to the challenge of chemically characterising hairy roots in this study, using the transgenic roots as a laboratory model. Establishment of the so called 'green hairy roots' may potentially meet the goal of up

regulating the essential secondary metabolites. Green hairy roots are known to produce certain metabolites that are normally synthesised in green parts of the plant (Giri and Narasu, 2000). Use of elicitation may further assist with increasing the levels of key secondary metabolites.

Several contributions have been made from this work which may assist with use of these plants in a phytopharmaceutical production line through maintaining elite chemotypes *in vitro*, assisting with breeding programmes aiming at generating new or improved traits. *In vitro* manipulation of culture conditions may clarify the effect of abiotic and biotic conditions on secondary metabolism; variants generated *in vitro* may acquire epigenetic changes which induce biochemical changes, upregulating key metabolites. The development of a transformation system for *S. runcinata* (**Figure 6.2**) is an important platform for future genetic manipulation of this species. This in itself is a major achievement as genetic manipulation of non-model species may be difficult using *Agrobacterium* as a vector. Overexpresson of key genes involved in secondary metabolism holds promise for generating higher levels of pharmacologically important bioactives. Production of hairy root cultures also provides another tool to study secondary metabolism in a non-model species in our laboratories. Overall, this study benefits the socioeconomic drive to utilise biotechnology as an income generator in South Africa, contributing to the number of African plants which have been studied for the first time in this way.

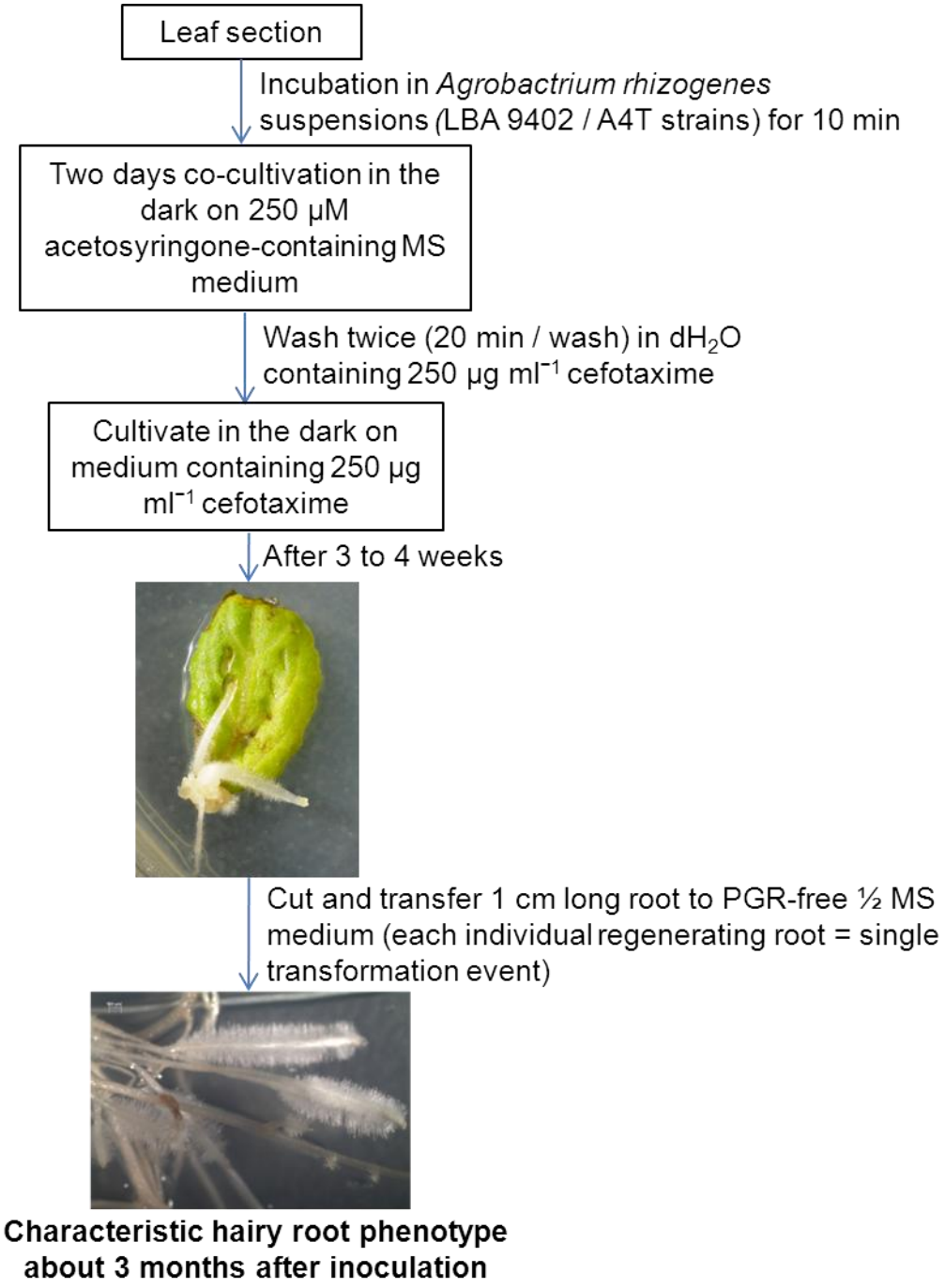

Figure 6.2 Optimal protocol developed for transformation of *S. runcinata* with *A. rhizogenes* LBA 9402 and A4T strains.

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Appendix[1]

Medium preparation

MS medium (Murashige and Skoog, 1962)

Components	Amount (mg L^{-1})
Macronutrient salts	
$MgSO_4.7H_2SO_4$	370
KH_2PO_4	170
KNO_3	1900
NH_4NO_3	1650
$CaCl_2.2H_2O$	440
Micronutrient salts	
H_3BO_3	6.2
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
KI	0.83
$FeSO_4.7H_2O$	27.8
$Na_2EDTA.2H_2O$	37.3
Organic supplements	
Thiamine-HCl	0.5
Pyridoxine-HCl	0.5
Nicotinic acid	0.5
Myo-inositol	100
Glycine	2
Sucrose	30

Note: Dissolve in distilled water and amend the pH to 5.7-5.8 with 1 M NaOH or 1 M HCl. The term of ½ MS refers to half the concentration of all MS chemical constituents. To amend the medium with phytohormones, add BA/IAA prior to changing the pH.

Appendix[2]

Headspace SPME GC-MS chromatogram

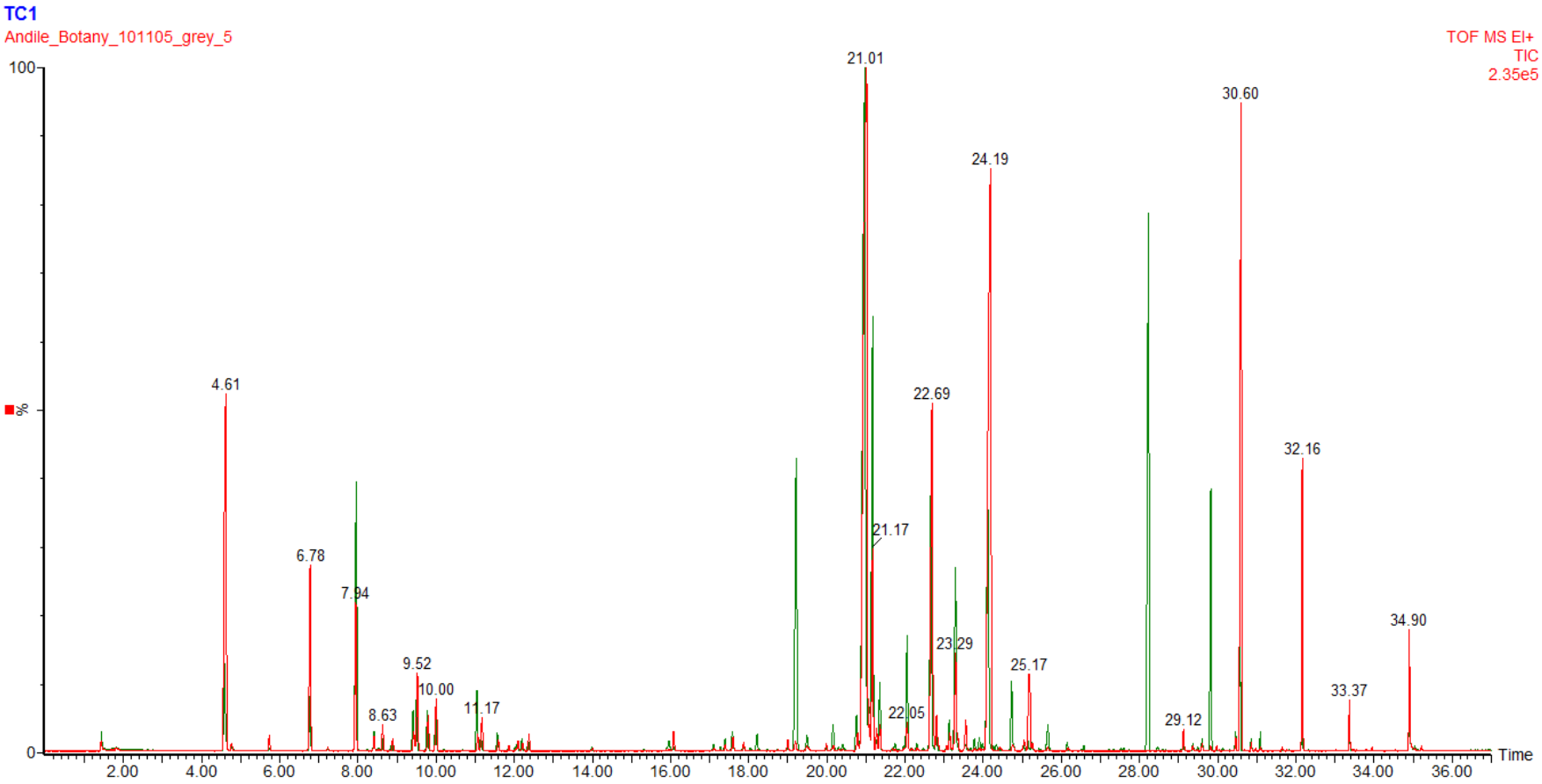

Figure A Volatile chemical profiles of tissue culture (red) and glasshouse (green) denoted by intergrated chromatograms.